Immunological response profiles to salmon lice infections in Atlantic salmon

Modulation by nutrition and selective breeding

Philosophiae Doctor (PhD) Thesis

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Contents

Acknowledgements 1

Summary 3

Sammendrag 6

List of papers 9

Abbreviations 10

1. Introduction 11

1.1. Current salmon lice situation in Norwegian aquaculture 11

1.1.1 Chemical control of salmon lice in Norwegian aquaculture 12

1.1.2 Husbandry, management and technical innovations against lice 14

1.1.3 Lice impact on wild salmonids 16

1.2. Life history traits of salmon louse and its fish hosts 17

1.2.1 Specifics of Atlantic salmon life history traits in comparison to Pacific salmonids 17

1.2.2 Salmon louse life cycle, feeding habits, communication and reproduction 20

1.2.3 Immunomodulation of the host by lice 25

1.3. Host responses to lice 26

1.3.1 Local skin tissue responses following *L. salmonis* infection 26

1.3.2 Immune responses 30

1.3.3 Systemic (physiological) responses to lice infection 42

1.4. Biological approaches for salmon lice control 45

1.4.1 Vaccines against lice 46

1.4.2 Selective breeding for increased resistance against lice 47

1.4.3 Anti-lice functional feeds 51

2. Objectives 57

3. Summary of Papers 58

4. Methodology 61
4.1. Fish experiments and study designs 61
4.2. Selection of tissues for various analyses 62
4.3. Microarray technology 65
4.4. qPCR 65
4.5. Histology and Immunohistochemistry 67
5. Results and Discussion 69
5.1. General discussion 69
5.2. Host responses during the copepodid and chalimus stages 71
5.3. Protection mediated by selective breeding 72
5.4. Protection mediated by glucosinolates-based functional feeds 75
5.5. Integrated pest management control 77
6. Main conclusions 79
7. Future perspectives 81
References 82
Errata 108
Scientific papers 111

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Summary

Infections with the salmon louse *Lepeophtheirus salmonis* (*L. salmonis*) represent one of the most important limitations to sustainable Atlantic salmon (*Salmo salar*) farming today. The parasite exerts negative impacts on health, growth and welfare of farmed fish, and there are concerns of the impact on wild salmon populations. Current control relies heavily on the use of a few chemical in-feed or bath treatments and an increasing number of reports states their reduced efficiency. Increased mortalities during treatments are also frequently reported. The narrow treatment repertoire is also a consequence of the difficulty in developing novel anti-parasitic chemical treatments that are efficient against lice, environment-friendly and posing no risk for the consumers. Functional feeds and selective breeding are considered two promising alternative approaches for management of salmon lice infections, and understanding the molecular basis of protection against lice is expected to help in their successful application in Atlantic salmon aquaculture.

The overall aim of this PhD study was to characterize responses to salmon lice infections and explore the possibility to modulate these by nutritional components and selective breeding. This required a basic understanding of the host responses to lice. The first study (paper I) investigated host gene expression responses at two skin locations, which are preferential sites for lice attachment and feeding activities; the scaled skin behind the dorsal fin and scaleless skin from the top of the head. Responses were studied before and during *L. salmonis* copepodid infection (4 days post infection (dpi)) and moult from copepodids to chalimii (8 dpi). Significant differences in basic transcript levels (constitutive expression prior to infection) were found between the two skin sites, suggesting a non-homogenous distribution of immune components across the skin of the fish. Immunohistochemistry was used to study *in situ* localization of MHC class II$^+$, Mx$^+$ and CD8α$^+$ cells. MHC class II$^+$ cells were condensed or diffuse in appearance and distributed evenly in the epidermis. CD8α$^+$ and Mx$^+$ cells tended to congregate to specific anatomic locations, especially apically along the *stratum basale,* which suggests this area is an important immunological region in epidermis. Furthermore, a small but significant increase in number of CD8α$^+$ cells was found. A temporal upregulation of most screened genes at both skin locations, including markers of innate, Th1/Th17 and Th2 responses revealed that the fish respond to infection with young stages of lice (copepodids) by mounting a mixed inflammatory response. At the chalimus stage however, paper

II found host immune gene expression responses inversely correlated to number of lice in the fish with the highest lice burden. Paper II studied responses in fish belonging to high lice resistant and low lice resistant families. The ranking of resistance/susceptibility was accomplished through calculated breeding values of lice density based on information of weights and lice counts of around 5000 Atlantic salmon individuals. These fish originated from 150 families, and all fish were experimentally challenged with salmon lice in two consecutive full-sibling trials. A subdivision of high resistant and low resistant fish by lice number (> 10 or < 10 lice per fish), made it possible to study the effects of lice burden on gene expression profiles apart from family background. Skin from the site of attachment was sampled for gene expression studies and histology. Histology revealed that the low resistant fish had larger mucus cells and thicker epidermis, likely related to the higher lice burden. Gene expression responses in infected skin from the high and low resistant families were compared by multivariate statistics to understand the relationship between lice load, family background and immune gene expression. Expression patterns were influenced both by genetic background and by the number of attached parasites. High resistant fish were better at resisting lice-induced suppression of both Th1 and Th2 related gene markers. This coincided with a 36 % reduction in lice counts compared to the low resistant fish selected for analyses. Low resistant fish however showed lower expression of most immune genes. It was thus shown that the genetic background of the fish impacts on the expression of immune genes associated with protection against salmon lice infection. Functional feeds may also increase resistance to salmon lice. Glucosinolates (Gls) and their main secondary derivative isothiocyanates (ITCs), are produced in plants from the Brassicaceae family as defence against herbivores, and beneficial effects in vertebrates are related to their antioxidant and detoxifying properties, and effects on cell proliferation and growth. In paper III, two dosages of Gls incorporated into the feed of non-infected and infected fish resulted in activation of interferon responses in skin, observed by oligonucleotide microarray technology and qPCR. Genes up-regulated in the infected fish fed Gls also included type 1 pro-inflammatory factors, antimicrobial and acute phase proteins, cytokines from both Th1 and Th2 pathways, extracellular matrix remodelling proteases and iron homeostasis regulators. These gene expression changes coincided with significantly ($P < 0.05$) lower *L. salmonis* numbers in both Gls fed infected groups. Not-infected and infected fish fed control feed were also included in the analyses. Genes involved in muscle contraction, lipid and glucose metabolism were found

more highly expressed in the skin of infected control fish. Results from paper III suggests that Gls modulate the local immune response in skin coinciding with lower number of salmon lice after experimental infection. Paper IV addressed the systemic effects of salmon fed various levels of Gls without or in combination with *L. salmonis* experimental infection. Oligonucleotide microarrays were also here used to profile responses to lice and Gls while qPCR was used to validate microarray results and study expression patterns of other genes of interest. Transcriptome profiling suggested activation of antioxidant and detoxification mechanisms in liver, muscle and distal kidney in response to the high content of Gls in feed. In all three tissues, activation of genes from Phase 2 detoxification was predominant, which is also reported in a number of *in vitro* and *in vivo* studies of Gls/ITCs exposed mammals and cells. Reduced growth was observed in infected fish fed high and moderate doses of Gls, a negative effect that was also reflected in a reduced liver size and reduced liver steatosis. A follow up study with lower inclusion levels of Gls showed no negative effects on growth. Increased expression of genes involved in pro-fibrotic and tissue-remodelling responses was found in distal kidney in fish fed the highest dose of Gls. However, biochemical plasma profiling of tissue-damage markers was not different to the fish fed control feed. Furthermore, regulation of genes involved in iron metabolism was seen in all three tissues, in line with recent studies suggesting that iron sequestration mechanisms may be an effective anti-parasitic strategy. Findings in this study encourage future use of GLs-based feeds due to their beneficial health effects on the expression of genes with detoxifying and iron-regulatory roles in multiple fish tissues. However, further refinement studies are required to better define the dose that promotes favourable anti-lice effects without posing a negative impact on growth or organ functions.

The results generated in this thesis provide a better understanding of the interactions between *L. salmonis* and Atlantic salmon post infection. Promoting the local immune responses, especially interferon and T helper cell responses in skin through selective breeding and functional feed, coincides with better protection against infection. Reduced effects of lice chemotherapeutants are becoming a big problem for farming of Atlantic salmon and results from this thesis lay ground for developing alternative strategies for lice management and control.

Sammendrag

Infeksjoner med lakselus (*Lepeophtheirus salmonis*) er en av de største utfordringene i dagens oppdrettsnæring, spesielt relatert til påvirkningen på dyrevelferd, resistensutvikling av de antiparasittære midlene brukt, økt infeksjonstrykk på villfisk, redusert vekst og slaktekvalitet. I dag kontrolleres lusenivået i all hovedsak ved hjelp av medisiner, som kan medføre økt mortalitet i seg selv. Behandlingsalternativene er begrenset, fordi det er vanskelig å utvikle nye medisiner mot lus som er både effektive, miljøvennlige og trygge for konsumentene. Behovet for å finne alternative strategier for å håndtere lakselusproblemet for fremtiden er stort, der både funksjonelle fôr og selektiv avl regnes som lovende alternativer. Dette krever en god forståelse av de molekylære responsmekanismene hos atlantisk laks ved lakselusinfeksjoner.

Den første studien (artikkel I) hadde som mål å studere den tidlige vertsresponsen ved eksperimentell *L. salmonis* infeksjon hos Atlantisk laks, ved to hudlokalisasjoner som lusa foretrekker (med og uten skjell). Dette ble gjort ved bruk av qPCR, før infeksjon og 4 og 8 dager etter infeksjon med 70 kopepoditter per fisk. I tillegg ble det brukt immunhistokjemiske teknikker for å studere cellemorfologi og distribusjon av MHC klasse II$^+$, CD8$^+$ og Mx$^+$ celler i huden. Disse to tidspunktene sammenfaller med henholdsvis kopepodittstadiet og skallskiftet til chalimus. Basale transkriptforskjeller mellom de to hudlokalisasjonene ble funnet, som kan bety at laks har en ikke-homogen distribusjon av immunkomponenter i hud. Det ble observert en oppregulering over tid for de fleste immungenene, inkludert markører for både det medfødte immunforsvar, Th1, Th2 og cytotoksiske T celler. I tillegg ble det funnet MHC klasse II$^+$ celler jevnt fordelt i epidermis, mens CD8α$^+$ og Mx$^+$ celler ble funnet i spesielle anatomiske lokalisasjoner i huden, som apikalt for *stratum basale.* I tillegg ble det funnet en liten, men signifikant økning av CD8α$^+$ celler i huden. Studien indikerer at atlantisk laks responderer på infeksjoner med kopepoditter og tidlig chalimus med aktivering av et blandet inflammatorisk genutrykk. På den andre side, artikkel II fant genekspresjon av både Th1 og til en viss grad Th2 markører inverst korrelert med chalimuspåslag. Denne studien studerte hudresponser i fisk tilhørende høyresistente og lavresistente familier. Denne rangeringen av resistens ble utført ved bruk av kalkulerte avlsverdier for lusetetthet fra to påfølgende eksperimentelle luseforsøk av over 5000 atlantiske lakseindivider fra 150 familier. Histologi og

genekspresjon i hud ved lusas festested fra individer tilhørende de mest resistente og mottakelige familiene ble sammenlignet ved bruk av histologi og qPCR. I tillegg ble fiskene fra de høy og lavresistente familiene undergruppert avhengig av om de hadde mer eller mindre enn 10 lus, og multivariat statistikk ble utført for å forstå sammenhengen mellom lusepåslag, familiebakgrunn og genekspresjon. Signifikant økning i mucusceller og tykkere epidermis ble funnet i huden hos de lavresistente fiskene, sannsynligvis relatert til deres høyere lusepåslag. Multivariat statistikk avslørte at både genetisk bakgrunn og lusepåslaget påvirket genuttrykket. Høyresistente fisk evnet å motstå luseindusert immunsuppresjon bedre enn de lavresistente individene, illustrert ved gruppering med interferon, Th1 og Th2 markører. Dette sammenfalt med en 36 % reduksjon i lusepåslag sammenlignet med de lavresistente fiskene, som var i større grad antikorrelert til interferon, Th1 og Th2 markørene. Studien viste dermed at genetisk bakgrunn har stor betydning for genekspresjon og kan gi beskyttelse mot lus. Aktivering av interferonrelaterte reaksjonsveier sammenfalt med økt luseresistens også i artikkel III, der potensialet til glukosinolater som en del av biologisk kontroll mot lakselus ble undersøkt for første gang. Glukosinolater produseres i planter fra *Brassicaceae* familien, og er en del av plantens naturlige forsvar mot herbivorer. Gunstige effekter på utvikling av kreft og en rekke degenerative lidelser i humane og dyremodellstudier er rapportert som følge av glukosinolat eller dens spaltningsprodukt isothiocyanater - eksponering. Disse effektene er relatert til stimulering av antioksidant og avgiftningsmekanismer og påvirkning av celleproliferasjon og vekst. I artikkel III ble lusepåslaget signifikant redusert hos atlantisk laks fôret med glukosinolatberiket fôr, sammenliknet med laks fôret med kontrollfôr. Oligonukleotid mikromatriseteknologi sammen med qPCR muligjorde omfattende studier av genekspresjonsuttrykket i hud hos fisk fôret med høy dose av glukosinolater før infeksjon, og 5 uker inn i infeksjonen som sammenfalt med utvikling til preadult og adult lus. Ikke infisert og infisert fisk fôret med kontrollfôr ble også inkludert, for å kunne studere infeksjonsrelaterte responser og fôrelaterte responser - *per se*. Induksjon av IFN-relaterte gener i huden hos laks fôret med den høye dosen av glukosinolater var bemerkelsesverdig. I den infiserte gruppa med fisk fôret med høy dose av glukosinolater var også IFN-relaterte gener aktivert, i tillegg til gener som koder for antimikrobielle og akutt fase proteiner, proteaser involvert i ekstracellulær modellering og jernhomeostaseregulatorer. På den andre side var gener involvert i muskelkontraksjon, fett og glukosemetabolisme aktivert i huden til fisk fôret med kontrollfôr. Resultatene fra artikkel III antyder at fôring med glukosinolater

kan medføre aktivering av et beskyttende immunforsvar mot lakselus lokalt i huden til atlantisk laks. Artikkel IV undersøkte de systemiske effektene av fôring med glukosinolater. Her ble også oligonukleotid mikromatriseteknologi brukt til å profilere genutrykket i lever, muskel og distale nyre hos fisk fôret med glukosinolater, i tillegg ble qPCR brukt for å validere resultatene og for å undersøke utrykket til andre gener av interesse. Aktivering av antioksidant og fase 2 detoksifiseringsmekanismer i lever, muskel og distale nyre hos fisk fôret med høy dose av glukosinolater ble funnet, liknende gunstige effekter av glukosinolater er også rapportert i en rekke murine og humane studier. I distale nyre ble det funnet økt ekspresjon av gener involvert i pro-fibrotiske og vevsmodulerende responser. Det ble også observert signifikant redusert vekst, leverstørrelse og fettinfiltrasjon i lever hos fisk fôret med høye og moderate doser av glukosinolater. På den andre side ble det ikke funnet signifikante forskjeller i plasmanivåer av enzymer som indikerer vevskade mellom fisk fôret med høy dose av glukosinolater og fisk fôret med kontrollfôr. Vekstreduksjon ble derimot ikke funnet i en oppfølgingstudie der fisk ble fôret med enda lavere inklusjonsniver av glukosinolater. Ved videre bruk av glukosinolater bør en undersøke hvordan de positive effektene kan beholdes, uten at organfunksjoner påvirkes negativt eller fiskens vekst reduseres.

Resultatene i dette doktorgradsarbeidet tilfører en større forståelse av vert-parasitt interaksjonene mellom *L. salmonis* og Atlantisk laks. På transkriptnivå sammenfaller aktivering av IFN og T hjelpeceller responser med redusert lusepåslag i hud. Selektiv avl og funksjonelt fôr kan redusere lusepåslaget hos Atlantisk laks og er bærekraftige alternativer for håndtering av lakselusinfeksjoner.

List of papers

Paper I

Contrasting expression of immune genes in scaled and scaleless skin of Atlantic salmon infected with young stages of *Lepeophtheirus salmonis.*

Jodaa Holm H, Skugor S, Wadsworth S, Bjelland AK, Radunovic S, Koppang EO, Evensen Ø.
Developmental & Comparative Immunology. 2016, in Press.

Paper II

Difference in skin immune responses to infection with salmon louse (*Lepeophtheirus salmonis*) in Atlantic salmon (*Salmo salar* L.) of families selected for resistance and susceptibility.

Holm H, Santi N, Kjøglum S, Perisic N, Skugor S, Evensen Ø.
Fish & Shellfish Immunology. 2015 Feb;42(2):384-94.

Paper III

Dietary phytochemicals modulate skin gene expression profiles and result in reduced lice counts after experimental infection in Atlantic salmon.

Jodaa Holm H, Wadsworth S, Bjelland Osmo AK, Krasnov A, Evensen Ø, Skugor S.
BMC Parasites & Vectors. 2016;9:271.

Paper IV

Nutrigenomic effects of glucosinolates on liver, muscle and distal kidney in parasite-free and salmon louse infected Atlantic salmon.

Skugor S, Jodaa Holm H, Bjelland AK, Pino J, Evensen Ø, Krasnov A, Wadsworth S.
BMC Parasites & Vectors. 2016, in Press.

Abbreviations

APC	Antigen presenting cell
CD	Cluster of differentiation
CpG ODN	Cytosine- phosphodiester-guanosine oligodeoxynucleotide motifs
DEGs	Differentially expressed genes
DF	Skin behind dorsal fin
FF	Functional feed
Gls	Glucosinolates
HD	High dose
HR	High resistant
HS	Head skin
IFN	Interferon
IHC	Immunohistochemistry
IL	Interleukin
IPM	Integrated pest management
L. salmonis	*Lepeophtheirus salmonis*
LD	Low dose
LPS	Lipopolysaccharides
LR	Low resistant
MA	Microarray
NK	Natural killer
PAMP	Pathogen associated molecular pattern
qPCR	Quantitative polymerase chain reaction
SEPs	Secretory/ excretory products
SFN	Sulforaphane
SLRC	Sea Lice Research Centre
Th1	T helper cell 1
Th2	T helper cell 2
TLR	Toll like receptor

1. Introduction

1.1. Current salmon lice situation in Norwegian aquaculture

The success story of Norwegian salmon farming industry involves an advancement from a small scale production 40 years ago to becoming one of the most important national industries today. Norway is the world's leading producer of Atlantic salmon and salmon export amounted to 18.2 billion NOK during the first four months of 2016, an increase of 25 per cent compared to the same period last year (1). 2015 was a record year with salmon export totalling 47.7 billion NOK (2), corresponding to 1 234 200 tonnes of salmon (3). The aquaculture industry is providing economic opportunities and income for people inhabiting the rural areas of the Norwegian coastline, and salmon filets have become an important trademark of Norway. The industry is not without challenges. In 2015, 287 million Atlantic salmon were put to sea, and 46 million fish were lost during the sea phase period. This is mainly due to infectious diseases, handling and escapees (3). Salmon lice (*Lepeophtheirus salmonis, L. salmonis*), crustaceans belonging to the family Caligidae, have been a problem ever since the beginning of the Norwegian salmon industry (4, 5). Today, *L. salmonis* represents the most important parasitic threat to sustainable Atlantic salmon farming in Norway (3), and the impact on wild fish is worrisome. An experimental infection of more than 30 preadult lice may kill a salmon post-smolt because of osmoregulatory breakdown (6). In addition, studies show a range of subclinical host effects of lice infections, which can lead to reduced fish welfare, growth and increased susceptibility to secondary infections, as reviewed in (7). Costs of lice management/treatment have recently been calculated to exceed 4 billion NOK (440 million Euro) in 2014 (8). Current regulations require that farmers apply strict measures for controlling the level of lice infection and lice numbers are monitored at least every 14th day throughout the year and reported to the Norwegian Food Safety Authority (Mattilsynet). Actions are required if the lice levels exceed 0.5 adult female lice per fish (9), which is a challenge, as there are few medicinal alternatives on the market and an increasing number of reports of their reduced efficacy (10-12). The use of mechanical delousing is on the rise in Norway (13). However, there are reports of cases where the treatments, including chemical and non-chemical methods, result in mortality and reduced fish welfare related to stress, physical trauma and excessive handling (3, 13, 14).

1.1.1 Chemical control of salmon lice in Norwegian aquaculture

Historically, chemical intervention has been the most commonly used method to control salmon lice levels on farms. The total amount used to control salmon lice has risen from 218 kg in 2008 (kg of active substance, excluding hydrogen peroxide), to 6810 kg in 2012, 8403 kg in 2013, 12812 kg in 2014 and 12768 kg in 2015 (15). This increased use of chemicals goes far beyond the production increase of Atlantic salmon in Norway, suggesting a decreased efficiency of the chemicals used (16). Norway is the only country that has applied a nationwide surveillance program for resistance monitoring, which shows a widespread reduction of sensitivity in most counties producing farmed salmon (11). Bioassays for salmon lice are used for testing the sensitivity of the lice towards a chemical. Resistance (of lice) is based on reduced sensitivity in bioassays (12), but the World Health Organization's definition of resistance is more general, namely "development of an ability, in a strain of insects, to tolerate doses of toxicants that would prove lethal to the majority of individuals in a normal population of the same species" (17). Living lice are exposed to several concentrations of the chemical for a period of time and the results are read by counting the number of alive and immobilized/dead lice, as reviewed in (12, 18). Increased tolerance has been reported for most of the medicinal compounds used against salmon lice, [reviewed in (12)]. It has been proposed that the increased resistance to these substances in lice has been developed because of inadequate and limited treatment alternatives, insufficient drug dispersal and feeding procedures, and an over-dependency of single substances for lice control (12). This is due to difficulties in finding new and suitable anti-lice substances (19, 20), further complicated as national approval and consents of practical use for new medicines are challenging to get and commonly takes years. This provides ample opportunities for drug-resistance to develop in salmon lice. Resistance mechanisms may arise by mutations, where positive selection of these individuals happens due to fitness benefits by pressure from a control agent. The rate of selection is determined by the level of fitness benefit/cost, the frequency of the chemical used, and the biology of the parasite (20, 21). Genes posing survival benefits are passed from a generation to another, and the number of individuals in a population with these resistance genes may accumulate. Salmon lice populations have been shown to share genetic resistance material across the North Atlantic ocean (22), implying that we might face a situation (in the future) where most lice are resistant to the anti-para-

sitic compounds available on the market. Refugia are parasites that have never been exposed to anti-parasitic treatments. Sea lice refugia may exist on wild fish, and may delay the development of widespread resistance as these lice harbour the wild-type genes (12).

Understanding drug resistance mechanisms is vital for monitoring the development of parasite resistance (23). Chemicals used against lice includes a few major classes of drugs: organophosphates, pyrethroids, macrocyclic lactones, chitin synthesis inhibitors and hydrogen peroxide, and a few possible resistance molecular mechanisms have been elucidated in lice [reviewed in (12)]. Organophosphates have been used since the beginning of fish farming in Norway, and reduced efficiency was reported already in the early 90s (24). Resistance to this compound is currently widespread (11), and the mechanism of resistance, a Phe362Tyr substitution in the acetylcholinesterase gene was recently found. This affects the binding of azamethiphos at the active site, i.e. impaired access to the active site (25). Resistance mechanisms in lice for emamectin benzoate involve reduced expression of target sites (26).

Chemical intervention is executed through bath treatment or medicated feed. Bath treatment is used for bigger fish, or when immediate treatments are necessary. Fish are usually starved before and after treatment (27). The advantage of bath treatment is an even distribution of medicine irrespective of fish size, appetite and hierarchy. On the other side, bath treatments are labour intensive and with risk of re-infections since pens are commonly treated one at a time and spill of the active ingredient can negatively affect other susceptible organisms in the proximity (7). Bath-treatments require that the fish are treated in an enclosed system separated from the surrounding water by raising the net and enclosing it with a tarpaulin system. Well boats can also be used where all fish are pumped into the boat. Hence, oxygen needs to be added to the water to ensure suitable levels for the fish. As the fish are contained in a small volume without any water change, in bath-methods can be stressful and mortalities can be high, especially in smaller fish (27). Applying in-feed chemicals is less labour- or time-consuming for the farmer and have less stress impact on the fish compared to bath treatments (28), but poses a risk for toxicity or under-dosage of the fish, and intake can be affected by cage hierarchy and health status of the fish (7). In-feed treatments are costly and thus recommended to be used in smaller fish (27), also because the withdrawal period after treatment may conflict with the time of slaughter in bigger fish.

1.1.2 Husbandry, management and technical innovations against lice

Good and dynamic husbandry and management techniques are important for controlling salmon lice levels on farms. As a minimum, the salmon lice regulations states that each fish farming site should have a plan for effective control and management of salmon lice, a plan which should be coordinated with other neighbouring fish farms. This plan should at least cover a description of efforts to reduce the development of resistance, including coordinated transfer of fish to the sea and fallowing and synchronized lice treatments throughout the year. Routine monitoring is compulsory; number of salmon lice shall be counted at least every 7th day if the temperature is over or at 4 °C, and every 14th day if temperatures are below 4 °C. Furthermore, to protect the migrating smolts during spring, fish farms need to make coordinated efforts from March 5 to June 25 when lice numbers exceed 0.1 lice per fish, so called spring delousing (9). The production and survival of copepodids are temperature dependent (29, 30), and show fluctuations throughout the year (Fig. 1), which needs to

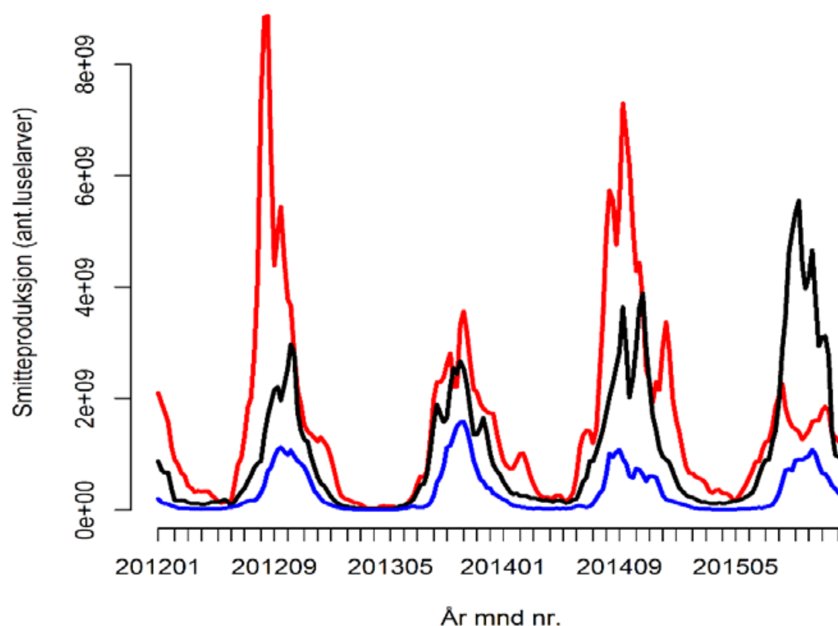

Fig. 1 The estimated weekly production of larvae on all fish farms in southern Norway (red line), mid-Norway (black line) and Northern Norway (blue line) in the period from January 2012 to January 2016. Used with permission from (3).

be taken into consideration when applying control measures against lice. The Norwegian Food Safety Authority (Mattilsynet) may also implement special regulations in areas along the Norwegian coast where lice counts are high, for instance synchronized fallowing and treatments within geographic zones, and destruction of salmon lice and eggstrings from water originating from well boats and slaughterhouses (31). Additional control measures may include growing of only 1-year class of fish, fallowing between production cycles, cleaning of nets and rotation of chemotherapeutants (32), coordinated stocking of single year classes and synchronized delousing during autumn and spring (27). Brooks described important oceanographic factors that should be involved in an IPM plan for salmon lice in the Broughton Archipelago, British Columbia (33) including recording of temperatures, salinity and currents and knowledge of migration patterns of wild salmon and location of salmon farms. This facilitates timely application of control measures corresponding with the migration of wild salmon.

There are numerous innovative technical solutions in the market for the management of salmon lice, and The Norwegian Food Safety Authority reports that the following are the ones most used in 2015 in Norway: hot water, tarpaulin skirts, optical delousing, 'snorkel' sea lice barrier technology, electrical skirt, and water assisted delousing (14). These methods need to be tested thoroughly for their efficiency to reduce lice number, and it is important that fish welfare be taken into consideration. The Norwegian Food Safety Authorities states in their most recent report on the salmon lice situation in Norway that reduced fish welfare and mortalities are often associated with using technical treatments against lice (13). *L. salmonis* larvae are normally found in the upper areas of the water columns at daytime (34), and technical solutions to prevent the farmed salmon to be present in the surface waters are considered a promising approach. Submerged sea-cage technology has been difficult to develop since it removes the possibility for salmon to regulate the amount of air in the swim bladder, which result in reduced welfare and growth (35). Including air filled domes that allow the fish to sip air might be one solution (36). The 'snorkel' sea lice barrier technology includes a cage with a ceiling that keeps the fish below the upper water columns, but access to air to fill the swimming bladder is ensured by having an enclosed tube that the fish can swim through to reach the water surface. Little or no, adverse effects on fish growth and fish welfare, and up to 65 % reduction in lice levels were reported in comparison to standard

cages (37). Other solutions for keeping the fish deep in the water is submerged lights and feeding to attract salmon to deeper waters, which also result in significantly lower salmon lice levels (38).

Inventions aiming at shielding the sea cages from outside lice transfer have also been investigated ("keeping the lice away from the fish"). Using planktonic shields where each cage receive filtered surface water is reported to give significant reductions in lice infection level, but requires substantial efforts to keep the nets clean, and lice may be able to access the cage from underneath the shield, or during situations where high waves flush lice over the shield (39). Another innovation is electrical skirts with wires pulled around the sea cage, that supposedly kill lice by transmitting electrical pulses (40). Solutions for mechanical delousing involves using either hot water (30-33 °C), brushes or waterjets to remove lice from the fish. 75-100 % lice reduction was reported using hot water (40). Optical delousing technology detects lice in seven milliseconds and removes lice from the fish within 100th of a millisecond using a pulse of light, i.e. laser technology (41), but the limited distance of action requires several laser delousers to be used in each cage (40).

1.1.3 Lice impact on wild salmonids

The interplay of sea lice, farmed and wild fish in the ocean is a topic that has received substantial attention from scientists, fish farmers, anglers, environmentalists and the media alike. The critical period is when salmon migrate from the native river towards the ocean to feed. In Norway, lice infection of wild salmonid populations is estimated through a national monitoring programme (42, 43). The migrating habits of different salmonids might affect when and where they are most at risk of being infected with salmon lice. Farming of salmon naturally increases the number of available, susceptible hosts for salmon lice. In the 1990s, heavily lice infected sea trout (*Salmo trutta*) were seen returning earlier to the native rivers than expected (44, 45). Atlantic salmon post smolts migrating through the fjords can suffer from high burdens of salmon lice, which may result in mortality (46). Fish farms are considered an important factor for the spread of salmon lice to wild salmonids in North America and Europe (47), but the magnitude of the negative impact is uncertain. Synchronized delousing of farmed fish (in early spring) coincided with low infection levels in migrating smolts in 2004 in Norway (48). In 2008, high infection levels on salmon and sea trout were found

throughout the Norwegian coast, which indicated that synchronized delousing was not sufficient to reduce the infection pressure on wild fish (42). In the summer of 2015, increased mortality of salmon post smolts and sea trout due to salmon lice infections were reported in certain fjords of Norway (43). The approaches commonly used to investigate lice infection rates and marine mortality of wild salmon are affected by how easy the fish are caught, time and place and the fishing equipment used, and only fish surviving the infection will be included (49). Monitoring of salmon lice levels in Norway through trawls show large variations in salmon lice level abundance on fish (46). Another approach (treat-release) involves treating smolts against salmon lice, releasing them, and comparing returns of treated and untreated fish, anti-lice treatment was found to increase number of Atlantic salmon returning from the sea (50). Another study using treat-release methods found that salmon lice infections were associated with an increased age of the returning Atlantic salmon in Norway (51).

1.2. Life history traits of salmon louse and its fish hosts

Parasites are believed to play an important role in the evolution of life-history traits of their hosts, due to their negative effects on host growth, fecundity or survival. The host may answer by shaping their own life history, to maximise their survival and fecundity. The host immune system is an important part of the host-parasite relationship, but immune resistance is energy demanding and possibly has to be a trade-off against growth and reproduction (see more below).

1.2.1 Specifics of Atlantic salmon life history traits in comparison to Pacific salmonids

The Atlantic salmon is an anadromous teleost of the Salmonidae family. Young salmon are hatched under the gravel of riverbeds and spend their juvenile stage in rivers, then migrate to the ocean, where they continue to grow, before returning to the native river to spawn. A few populations of salmon spend their entire life in fresh water, or stay in the sea water close to the river. Spawning in rivers take place from September to February. Hatching of eggs in a riverbed occurs during springtime, and the newly hatched fish of 15-25 mm length (alevins) depend on the yolk sack for 3-8 weeks before they start to feed. This stage is called fry, and are free-swimming and able to move up in the water column. The vertical stripes make the fry blend in with the environment, providing some protection against predators in

the river. Lack of food at this time point leads to starvation and death. The next juvenile stage is called parr (fingerling), easily recognized by the black spots on the sides of the fish. Depending on the temperature and availability of nutrients, at this stage fish remain in the river for at least one year before migrating to the sea. When the parr has reached a certain size, a physiological change called smoltification commences, which prepares the salmon for life in salt water. Smolts are usually of 10-20 cm in body length, weighing 10-80 g, with silvery sides, dark back and white belly. The Atlantic salmon smolt migration happens in spring and early summer. Atlantic salmon can spread to vast areas in the North Atlantic Ocean (52) as shown in Fig. 2. Salmon sexually mature after one year or more in the ocean, and return to

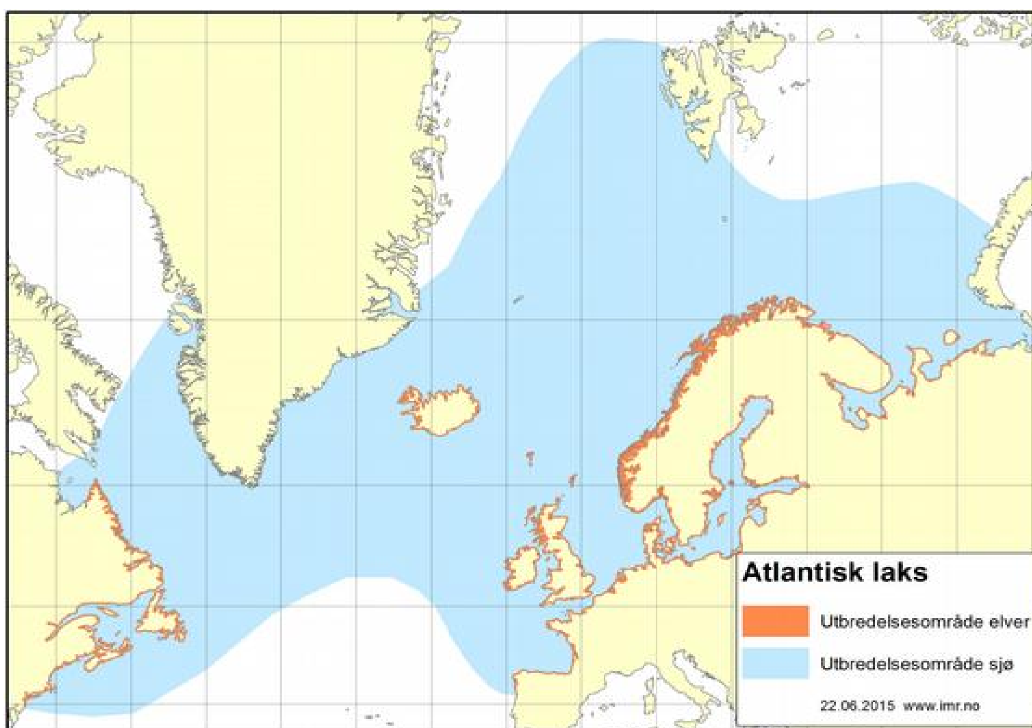

Fig. 2 The distribution of wild salmon in rivers (orange areas) and at sea (blue areas). Used with permission from Institute of Marine Research, Bergen, Norway (53).

its native river from May to October in Norway, a process named homing (53). During the sea phase, the size of the salmon can increase to 1-25 kg (45-135 cm total length), but some individuals may be over 30 kg. The feeding habits of salmon change during the lifetime; in freshwater, the fry mainly feeds on zooplankton, insects and plants depending on the size, and the parr eats aquatic insects, worms, crustaceans, amphibian larvae, fish eggs and also young fish. In the ocean, the feed can consist of plankton, small fish, including herring and lantern fish (52).

Susceptibility to *L. salmonis* infection vary among salmonid species (54). Coho salmon (*Oncorhynchus kisutch*) and pink salmon (*Oncorhynchus gorbuscha*) are highly resistant, while Atlantic salmon, rainbow trout (*Oncorhynchus mykiss*), chinook (*Oncorhynchus tshawytscha*), and chum salmon (*Oncorhynchus keta*) are among the susceptible species (55-60). Lice mature more slowly on resistant compared to susceptible species (57, 61). The production of *L. salmonis* eggs and larval survival is significantly reduced on atypical hosts such as cod (*Gadus morhua*) compared to Atlantic salmon (62). The range of resistance among salmonids against lice could be a trade-off between strong immunity towards the parasite versus development and growth (54). Age-dependent development of resistance to *L. salmonis* has been shown in pink salmon that enter the ocean at a very small size lacking scales. Pink salmon develop resistance to lice when they are between 0.3 and 0.7 g in size (63), likely related to maturation of osmoregulatory functions, skin and immune system (63, 64). It is commonly observed that larger salmon harbour higher infection levels (65-67). The reason for this is not known, but can include preference for an individual with a high condition factor, as host fitness may be positively correlated with parasite survival and fecundity (68). When sexual maturation commences, there is a decreased resistance to salmon lice in pink salmon (54) and adult migrating pink salmon are observed carrying high lice burdens (69). For Atlantic salmon, experimental and field data showed that resistance greatly increases in sexually mature fish (70). On the other side, sexual maturation is considered a constraint in fish farming, which may conflict with the desire for rapid growth and low production costs. In fact, the natural lifecycle is substantially modified in farmed Atlantic salmon. The production lasts for 10-16 months in freshwater and 14-24 months in seawater, which results in a life expectancy of a farmed salmon of 24-40 months. Brood stocks are harvested for eggs and milt, which are mixed and incubated in freshwater for about 60 days. The newly hatched fry is transported from the hatcheries to larger containers once it starts to feed (71). The parr lives in freshwater until they are 60-100 g (72), which can take 10-16 months (71). The growth of juveniles and time of smoltification can be shortened to 6 months by light manipulation. To even the harvesting volume, smolts are transferred to sea water twice a year in Norway (73). The production phase at sea can last from 14-22 months until the fish reaches a slaughter weight of 4-6 kg (71).

1.2.2 Salmon louse life cycle, feeding habits, communication and reproduction

The salmon louse belongs to the Caligidae family, which is a part of the phylum Arthropoda. Two subspecies of salmon lice were recently identified; *Lepeophtheirus salmonis salmonis* (Atlantic) and *Lepeophtheirus salmonis onchorynchii subsp. nov* (Pacific) (74). The life cycle of salmon louse is complex and consists of eight life-stages (Fig. 3) divided into a planktonic phase and a parasitic phase. The stages are: two planktonic nauplii, one infective copepodid, two sessile chalimii and mobile stages that comprise two pre-adult and the reproductive adult stage. Every stage is separated by moulting in which the exoskeleton is shed (75, 76). In addition, a new frontal filament is produced for each chalimus moult (77). The rate of development, generation time and fecundity of *L. salmonis* varies with temperature (78). The generation time of salmon lice has been estimated to be in the range of 52 days at 10 °C for females and 40 days for males in Atlantic salmon (79). An additional 9 days should be added for egg production (78). The reproductive potential increases with temperature as a result of decreased generation time and increased reproduction (29). Heuch *et al*. found adult females producing 11 pairs of eggstrings for 191 days at 7.2 °C. The eggs tended to be

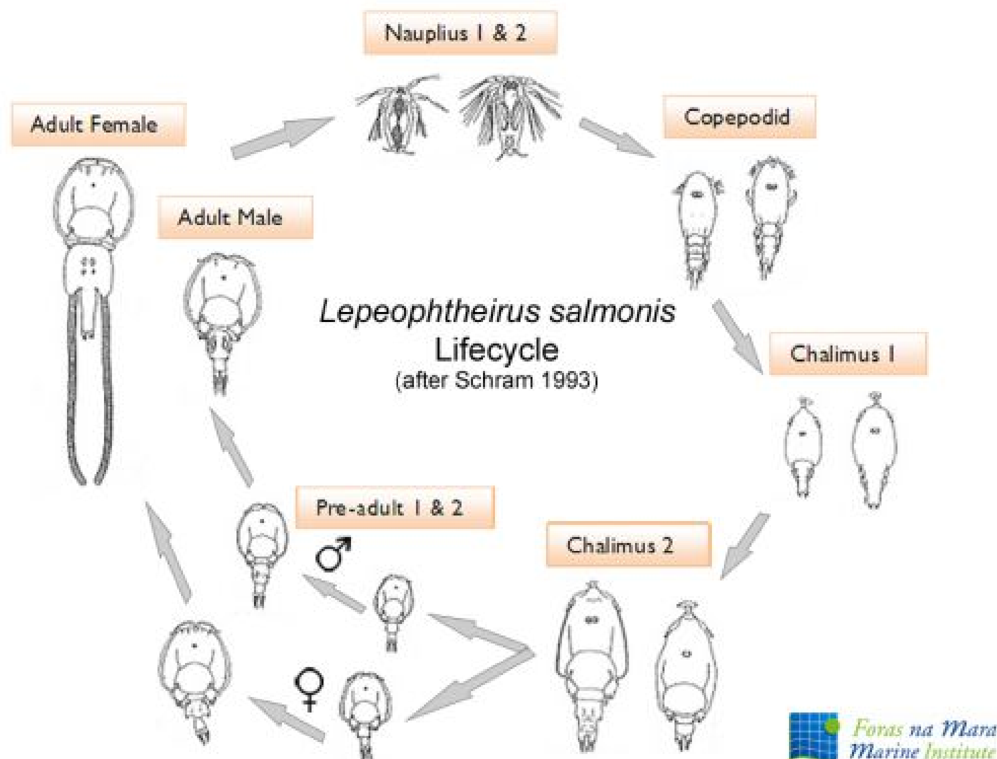

Fig. 3 Representation of the life cycle of *L. salmonis*. The figure is from (83) and adapted by the Marine Institute of Galway. Used with permission.

smaller and less viable at this temperature compared to a higher temperature (12.2 °C) (80). The time of hatching can range from 45.1 days at 2 °C to 8.7 days or 9 days at 10 °C (79, 81). The nauplii and copepodids are lecithotrophic, meaning that they rely on their yolk reserves for energy and survival. Level of energy reserves affects the ability to infect the host (82). The nauplii stages are 0.5-0.6 mm long (83, 84) and drift passively through the water masses. Dispersal models predict that lice can be transported up to 30 km (85). Tully summarized the current literature and found that the noninfective nauplius I and II stages last for 223.3 h at 5 °C; 87.4 h at 10 °C; and 50.0 h at 15 °C (78). The copepodid is 0.7 mm long (83) and non-feeding, despite the presence of a functional gut with external mouthparts (86). The cope-podids show searching and probing behaviour on the host, before commencing the settle-ment behaviour (87). By using the second antennae, which have hook-like structures, and grasping with the maxillipeds (87), the infective copepodids attach to the host. The second antennae embed into the epidermis and breach the host basement membrane (87, 88). Mechanosensory and chemosensory elements in the antennal system positioned proximally on the louse are likely involved in sensing currents and chemicals in the milieu (89, 90), as the copepodid stage show signs of phototactic (34, 89, 91), rheotactic (92) and chemotactic (93) behaviour. Semiochemicals aid lice in finding the correct host (93, 94) and in finding correct partner for mating (95, 96). Removal of the distal tip of the antennae reduces the infection success (96). Filament material for the frontal filament is present internally in late copepodid stages, and external frontal filament can be seen at the chalimus stage (77). Co-pepodid host settlement show correlation with temperature and salinity (97). The develop-mental times for copepodids from hatching vary with temperature, 9.3 days at 5 °C, 3.6 days at 10 °C and 1.9 days at 15 °C (79). The duration of the copepodid stage was in the same study calculated to be 10 days at 10 °C, but the duration also depends on salinity (79, 97). The subsequent chalimus stage attaches to a host by the elongated frontal filament (77, 87), which is attached to the basal membrane of the epidermis by an amorphous basal plate (88) and restricts the area of feeding. The frontal filament is replaced every chalimus moult (77). The chalimus body shape gradually changes to a morphology similar to the adult stages. Chalimus I is 1.2 mm in length, whereas chalimus II is 2.2 mm long, approximately. The adult stages move freely on the host skin, and their body consists of four main parts; cephalotho-rax, thoracic segment, genital segment and abdomen (84). The preadults are readily distin-guished sexually and by size: preadult I males are 3.4 mm, preadult II males are around 4.3

mm, while preadult I females are 3.6 mm and preadult II females 5.2 mm (83). Adult males are 5-6 mm (83). Females are larger, 8-11 mm in length (83) and have a triangular genital segment, compared to the barrel-shaped male segment (91). Ovaries and testes are present at the postchalimus stages, and fully developed at the adult stages (98). Preadults are susceptible to water currents, especially the males may drop off with increasing water flow (99), and adults may also change hosts (96, 99). Adult females can survive up to 191 days at 7.2 °C, and produce 11 pairs of egg strings (80), a new set of egg strings can be produced every 10 days (100). Lice feed on mucus, skin and blood, although the importance of each dietary component in different life stages stage is not well understood. The adult females are particularly associated with blood-feeding (101), but it has been suggested that blood is eaten opportunistically, as a result of damage to skin capillaries following the skin grazing activities (86).

Salmon lice seem to have preferred sites of attachment on the fish body (86), which may be related to local currents and how easily the copepodid may attach to a certain area (87). Lice are also able to wedge below scales (102), which can provide protection against the water currents. In wild sea trout, chalimii were reported on fins (103), while later stages settled on head and lateral body surface (104) and on the dorsal surface (105). Lice induced damages are reported to occur on fins and dorsally on wild sea trout (103). In wild Atlantic salmon, chalimii were found on fins and the body (106), preadult and adult females was found in proximity to the anal fin and on the dorsal midline between the dorsal and caudal fins, and adult males on the sides of the head and along the dorsal midline between the head and the dorsal fin (107). Apart from this, there are some variation as to where different stages and lice associated lesions are located on the body surface, fins or body (106), depending on development stage, water temperature, salinity and size of the fish (Table 1). Light, host velocity and salinity may influence copepodid distribution as shown for experimentally challenged Atlantic salmon (108).

Anatomical location	Lice stage	Type of trial	Reference
Gills	Cope-podids	Experimental challenge	(60)
Fins > gills > head > body	Cope-podids	Experimental challenge	(108)
Epidermal reactions on the dorsal side of the fish	Cope-podids	Experimental challenge	(6)
Gills > fins > head at water temperature of 6.9 °C (± 1.3 °C)	Cope-podids	Experimental challenge	(97)
Fins > gill at water temperature of 11.7 °C (± 0.6 °C)	Cope-podids	Experimental challenge	(97)
Gills > fins at salinity of 24 ‰	Cope-podids	Experimental challenge	(97)
Body > fins at salinity of 34 ‰	Cope-podids	Experimental challenge	(97)
Gills > fins > body (operculum)	Cope-podids and chalimus	Experimental challenge	(79)
Lesions observed in perianal areas and fins	Chalimus	Experimental challenge	(88)
Fins > body > gills	Chalimus	Experimental challenge	(87, 109)
Head, operculum and dorsal (posterior) part of the body	Post-chali-mus	Naturally infected fish from sea cages	(99)
Head and operculum (smaller fish of 24-44cm length) and dorsal part of the body. Adult females occupy the post-anal area in large fish (44-75 cm length)*	Post-chali-mus	Naturally infected fish from sea cages	(99)
Similar to *, but lower levels on operculum, and higher levels in	Postchali-mus	Naturally infected fish from outdoor tanks	(99) (Fig. 4)

the posterior part of the back and at the post-anal area (adult females)			
Adult males on the head and near dorsal fin. Females posterior to the adipose fin and the anal fin (females)	Preadults and adults	Experimental challenge	(109)
Lesions found dorsally on the head, or behind the dorsal and anal fins	Preadults and adults	Experimental challenge	(102)
Lesions on head and operculum	Preadults and adults	Experimental challenge	(109)

Table 1 Summary lice location of different development stages on Atlantic salmon.

Fig. 4 Adult salmon lice infecting area around the anus of a salmon. Used with permission from Institute of Marine Research, Bergen, Norway. Photo: Lars Hamre.

1.2.3 Immunomodulation of the host by lice

Parasites typically excrete or secrete substances that result in immunomodulation of their host. It is well established that this strategy supports successful infection of mammalian hosts. Immunomodulatory substances can ligate, degrade or interact with the host immune cells and their signalling pathways, and are named secretory/excretory products (SEPs), since it is often not known whether the compounds are passively secreted or actively excreted (110, 111). The complex parasite-host interactions are a competition between the anti-parasitic defences of the host and the host immune evasion strategies of parasites. Molecules associated with excretory/secretory products of lice, which are suspected to act as immunomodulators, include proteases, cathepsin L and prostaglandin E2 (PGE2) (112-116). Four PGE2 receptors are found in Atlantic salmon (117), and PGE2 containing SEP fractions were shown to inhibit the expression of the pro-inflammatory cytokine *il-1β* and the *major histocompatibility class (mhc) I* and *II* genes (118). Similar immunosuppressive activities are also reported in murine models. PGE2 from ticks inhibits cytokine production and reduces dendritic cell maturation and antigen presentation (119). In *L. salmonis* studies, inhibition of *il-1β* expression in Atlantic salmon head kidney cells was also observed after exposure to fractions of putative PGE2 free excretory/secretory products derived from *L. salmonis*, thus indicating the existence of other immunomodulatory substances (120). Trypsin-like proteases suspected of being lice derived were found in infected Atlantic salmon mucus (115, 116) as early as 3 days dpi (115). A higher percentage of salmon lice secretes proteases after incubation with rainbow trout or Atlantic salmon mucus in comparison with coho salmon mucus (121). Incubation with prostaglandin E2 containing excretory/secretory products from dopamine treated adult lice induced phagocytic activity in Atlantic salmon macrophage-like SHK-1 cells (114). The same study also showed differences in macrophage activity among salmon species; macrophages from pink salmon showed higher respiratory burst following stimulation with lice secretions compared to Atlantic salmon. On the other side, macrophages from chum salmon had higher phagocytic index than Atlantic and pink salmon macrophages. Different macrophage responses among salmonids were also reported in (57, 122).

1.3. Host responses to lice

Lice infections induce a number of local and systemic responses in their hosts. Skin damages and reduced growth can be observed at high level of infestation in Atlantic salmon. Skin lesions may lead to loss of blood and serum proteins (6). Activation of stress responses with cortisol release is often reported (46, 123). Iron sequestration responses in resistant salmonids appears as an important contributor in the defence against lice (59). Studies have shown that many immune responses are suppressed at some point during the infection in the susceptible species, such as Atlantic salmon (55, 56, 124, 125). Immune responses in Atlantic salmon do not result in appreciable level of protection. The following chapters aimed at providing basic information on involved immune factors and processes, which will precede the literature review of immune responses to lice, which represents one of the most important topics in this thesis.

1.3.1 Local skin tissue responses following *L. salmonis* infection

The skin mucosa is the first line of defence against invading pathogens. The mucus membrane consists of epithelial cells and leukocytes. Most of the fish skin surface is covered by scales. Epithelial cells are non-keratinized and in close contact with the mucus producing cells in epidermis (Fig. 5). The mucus consists mostly of water and glycoproteins, mainly mucins, which give mucus the characteristic viscosity. The mucus of fish has important roles in respiration, osmoregulation, reproduction, locomotion, excretion and communication and defence against pathogens (126, 127). It is rich in proteins and carbohydrates, forming an intricate network between the hosts, commensal bacteria and invading pathogens. Immune defence substances in the teleost mucus include immunoglobulins (128-130), immunoglobulin receptor (130), antimicrobial peptides (131-134), lysozyme (135, 136), complement (129), lectins [reviewed in (137)], enzymes including proteases, lipases and polysaccharide-degrading enzymes (129), and iron binding transferrin (138).

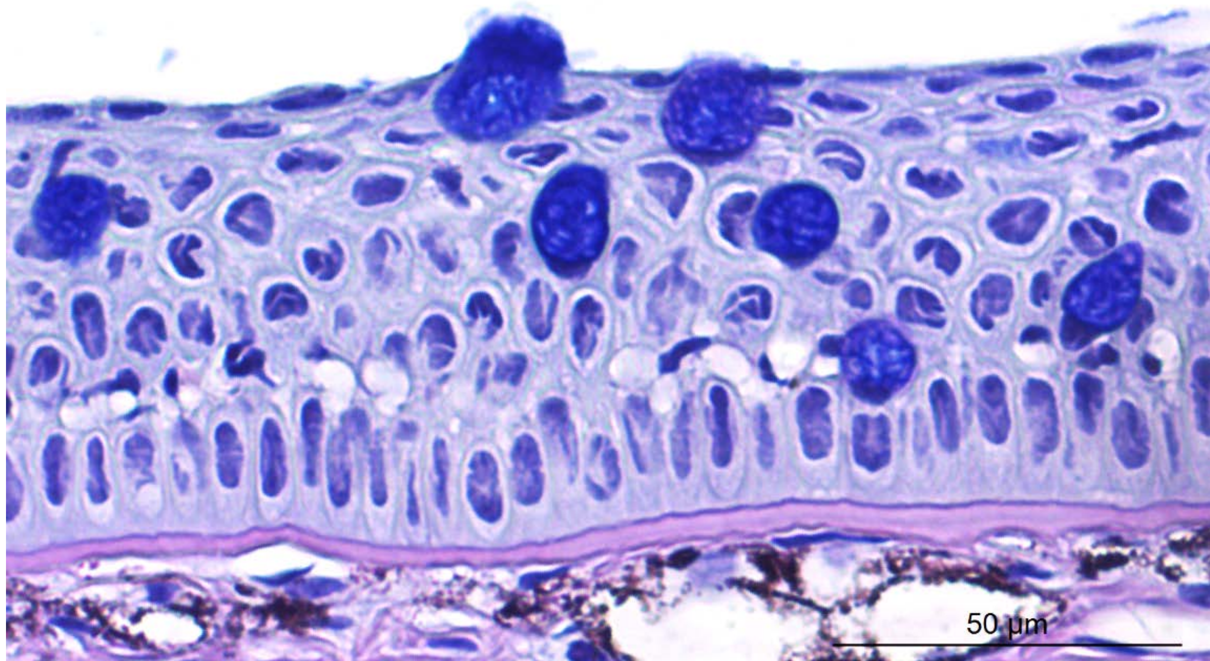

Fig. 5. Epidermis in skin of Atlantic salmon. Note mucus cells are stained blue with AB-PAS stain. From paper I.

There are several studies addressing salmonid skin responses to sea lice infections (55-57, 59, 60, 139-141). Lice-induced skin damages are dependent on the number and developmental stage of the parasite (86). In wild sockeye salmon, lesions can range from mild skin discoloration to skin lesions exposing the underlying musculature, resulting in increased mortality (142). The scull may be exposed in some cases (91). Copepodids cause a small black spot where they attach, likely because of melanocyte aggregation (6, 86). When the chalimus frontal filament is anchored to the skin, the feeding activities commences with mechanical disruption of the epidermis (88), which can result in fin ray exposure and extensive skin erosions on the dorsal side of the fish (103). Not surprisingly, area of damage on dorsal fin is positively correlated with increasing lice numbers, a mean of 140 chalimii caused damage to over 66 % of the trout fin surface in one study (105). Jones *et al.* reported lesions in wild Atlantic salmon following chalimus release to appear as an outer ring of pigmentation, with a depressed white core (88). The preadult and adult stages are able to feed over large host surface areas, as they are not attached by the frontal filament. The body of the parasite may work as a suction cup, facilitating undisturbed feeding activities (86), resulting in an oval ring around the area of attachment of similar shape as the marginal membrane of the cephalo-

thorax in experimentally infected fish (102). The activities of preadults and adults are associated with extensive host damages (Table 1). These are described as having grey edges and a haemorrhagic centre with substantial skin and scale loss. Lesions may occur on top of the head, between dorsal and adipose fin and ventrally close to the anal fin (102).

Changes in skin are also observed microscopically. One study addressed the local skin responses in Atlantic, chinook and coho salmon following experimental infection (approximately 30 *L. salmonis* copepodids) (60). Gill and fin erosions were observed in all species already at 1 dpi. The lice burden was significantly lower in coho at 5, 15 and 20 dpi than in Atlantic salmon. Lower lice burden in coho salmon was linked to a stronger local inflammatory cell infiltrate (mainly neutrophils) in gills, and epithelial hyperplasia in the fins, which could result in a total encapsulation of the louse. In contrast, attachment and feeding sites of chinook and Atlantic salmon showed erosion and fusion of secondary gill lamellae, and only mild inflammatory responses were observed in fins. Similarly, little dermal reaction was observed in the vicinity of the secondary antennae and the frontal filament of copepodids and chalimii infecting Atlantic salmon (88). As the basal plate of the frontal filament attaches to the basal membrane of the fish, the epidermis in the proximity of the filament may be lost. However, remnants of frontal filament material, nodular lesions with hyperplastic epidermis, macrophage infiltration and surrounding fibrosis were observed after chalimus release (88). On the other side, Jónsdóttir *et al.* reported a cellular response in close vicinity to preadult and adult parasites in Atlantic salmon skin, where the cellular infiltrate went down into the muscular tissue (102). In addition, Nolan *et al*. (143) reported pathological changes in Atlantic salmon skin away from the site of lice attachment already at 1 dpi, at an infection level of only 3-10 preadult and adult lice per fish. Skin responses included presence of necrotic keratinocytes, increased intercellular spaces and staining of desmosomes. The changes were positively correlated to the infection level. In gills, swelling of the gill lamellae and apoptotic chloride cells were already observed at the lowest infection level (3 parasites per fish) at 1 dpi. At 5 dpi, the gill Na+/K+-ATPase activity was significantly higher, and positive correlation was observed between the magnitude of leukocyte infiltration and infection level (143).

Mucus cell responses to lice infection have also been studied (56). Proteomic studies have identified a number of immune relevant molecules in fish mucus (144) and levels vary with

fish species (135, 145). These may lead to pathogen immobilization, inhibit pathogen cell surface binding, activate the host cellular defences and induce phagocytosis, which can ultimately result in the destruction of pathogens. However, changes in mucus compositions or amount might also be favourable for the parasite. Copepodids use semiochemical stimuli emitted from the skin and mucus of fish for detection of a suitable host (93-95, 146-149). Variation in host mucus composition might affect the parasite recognition and attachment process. Interestingly, it has been hypothesized that salmon resistant to the ectoparasitic monogenean *Gyrodactylus salaris* employ a starving strategy by reducing the proliferation of epithelial cells at the site of parasite attachment (150). A related parasite, *Gyrodactylus derjavini*, appear to select sites for feeding based on the density of mucus cells, a study showed that skin sites of rainbow trout with fewer mucus cells harboured more parasites (129). Genes encoding mucosal proteins were found to be differentially regulated in steroid hormone treated Atlantic salmon infected with lice (70). At the protein level, mucus composition is shown to differ in Atlantic salmon exposed to different anti-lice functional feeds (151). Finally, there are mucus related differences among lice resistant and susceptible salmonids (56). However, it is not yet clear if any of these previously reported differences play a role in protection against lice. Enzyme levels, including proteases also differ among salmonid species (140). Proteases are classified into serine, cysteine, aspartic and metalloproteases based on the chemical nature of the groups responsible for catalysis. Representatives of all categories are identified in fish mucus (145). These proteases can split bacterial proteins, and enhance production of other immune components, including complement (152), and immunoglobulin (153). One study compared the mucus protease, alkaline phosphatase and lysozyme levels as well as plasma lysozyme activities and histological parameters, i.e. epidermal thickness and mucus cell characteristics, in three salmonids, rainbow trout, Atlantic salmon and coho salmon. Atlantic salmon had the thinnest epidermis, the lowest distribution of mucus cells and lowest activity of mucus lysozyme and proteases (140). A follow up study investigated regulation of these skin parameters after experimental *L. salmonis* infection. The outer epidermis of Atlantic salmon was also in this study significantly thinner, with less mucus cells than rainbow trout or coho salmon. Furthermore, mucus cell hypertrophy was observed in skin from Atlantic salmon and rainbow trout at 1 dpi compared to not infected controls (57).

The same study also showed different mucus enzyme activities among *L. salmonis* infected salmonids. Despite Atlantic and rainbow trout harbouring higher lice burdens than coho salmon, rainbow trout had higher alkaline phosphatase levels and lysozyme levels than the other two species for most of the infection period (up to 21 dpi). Atlantic salmon mucus lysozyme activities and alkaline phosphatase activities increased significantly already at 1 dpi and 3 dpi, respectively, compared to not infected controls. In coho salmon, no differences in alkaline phosphatase activities were found, and lysozyme peaked only at 21 dpi, which made it difficult to understand the possible correlation between lice burden and enzyme regulation (57).

1.3.2 Immune responses

In addition to the physical barrier that consists of mucus, scales and the epidermis, the defence system of fish also involves cells from the innate and adaptive immune responses. Cells of the mammalian immune system originate from two types of stem cells, a common lymphoid progenitor that can develop into natural killer cells (NK cells), T cells and B cells and the myeloid progenitor that gives rise to monocytes and granulocytes such as neutrophils, eosinophils, basophils and mast cells. The innate lymphoid cells (ILCs) belong to the lymphoid lineage, but lack a B and T cell receptor.

The first step in combating a pathogen is *recognition*. Mucosal epithelial cells act as sensors for damage to the skin, which causes production of damage associated molecular patterns (DAMPs) that activate downstream inflammatory responses. Pathogen recognition receptors (PRRs), like Toll like receptors (TLRs) (154), also play a role in activating downstream inflammatory responses. Epithelial cells have antigen presenting properties (155), and secrete cytokines that stimulate the migration and activation of effector immune cells to the site of damage (156). Production of cytokines occur early after epithelial cell damage. Cytokines interleukin 1 β (IL1β) and tumor necrosis factor α (TNFα) can activate the early acute response, which is characterized by hepatic and extrahepatic production of acute phase proteins, including C-reactive protein, fibrinogen, lactoferrin, serum amyloid A and haptoglobin. These proteins form a systemic early defence against microbes, by acting as chemoattractant for immune cells, opsonins for phagocytes, iron binders and activators of the complement system (157). The net result of three pathways of complement activation is binding of C3b to the microbial surface, formation of the microbiocidal membrane attack complex

(MAC) and microbial destruction. The complement is also important for activation of B cell responses (158).
Phagocytosis is a fundamental mechanism to remove pathogens and/or cell debris, but also crucial for tissue homeostasis and remodelling (159). Phagocytes are activated by cytokines and microbial products, and in the activated state, these cells may phagocytose and kill pathogens by effectors such as NADPH oxidase derived superoxide, by production of nitric oxide (NO) by inducible nitric oxide synthase (iNOS) and lysosomal proteases (160). These compounds may be liberated in the extracellular space and cause collateral tissue injury (161). Neutrophils may also be involved in resolution of the inflammation by production of anti-inflammatory lipids (162).
Blood monocytes travel to inflammatory sites, where they infiltrate the tissue and differentiate to macrophages (163). Macrophages are key modulator and effector cells in the immune system, shown by the range of receptors they possess, including receptors to communicate with T cells and B cells. Macrophages secrete cytokines and present foreign antigens to T helper cells. They belong to antigen presenting cells (APCs). MHC class II molecules present antigens that are derived from endocytosed proteins in vesicles in APCs while MHC class I molecules present mainly cytosolic proteins (161). MHC positive cells may include professional APCs like monocytes/macrophages, dendritic cells and B cells, but also nonprofessional APCs, such as epithelial cells (164). Dendritic cells capture and endocytose microbial immunogenic antigens at epithelial surfaces and in mammals migrate to the draining lymph node to present the antigen on MHC molecule to T cells to promote antibody- and cell-mediated responses. They are often classified as classically vs. alternatively activated, based on two distinct phenotypes and functions they specialise in. The classical macrophages, also called M1, are activated by IFNγ, produce the reactive oxygen species (ROS) and the toxic nitric oxide (NO) from L-arginine by the inducible nitric oxide synthase enzyme. The alternative macrophages, also called M2, produce ornithine and urea from L-arginine by arginase and are activated by IL4 (165). These two types of macrophages have different activities; host defence, release of proinflammatory cytokines, and enhanced intracellular killing of pathogens (M1), or tissue healing and immune regulatory functions (M2) (166). However, the classification into classical and alternatively activated of macrophages is likely oversimplified (167). Murray *et al*. have suggested that the macrophage nomenclature in experimental studies involving *in vitro* work should encompass at least the source of macrophages,

definition of the activators, and a consensus collection of markers to describe macrophage activation, to ensure the reproducibility of the work (168).

Eosinophilic granulocytes are often associated with allergy and response to parasitic infections in mammals (161). It has long been proposed that eosinophils armed with parasite specific IgE may kill parasites by degranulation, but the importance of this function is not clear (169). The eosinophil peroxidase and major basic protein are released after eosinophil degranulation. Mice deficient in these suffer from higher worm burdens (170).

T cell receptor (TCR) chains express either αβ or γδ, but all have cluster of differentiation 3 (CD3), important for signal transduction (161). In mammals, the $\gamma\delta^+$ cells are innate-like cells, mainly present in epithelial and mucosal areas, and are activated without involvement of major histocompatibility complex (MHC) molecules. αβ-T cells can be divided into cytotoxic T cells or T helper (Th) cells, distinguished by the expression of the membrane bound glycoproteins CD8 or CD4, respectively. These molecules are also co-receptors for the TCR, stabilizing the interaction with the MHC and enhancing TCR activation and signalling. Naïve T cells are activated by recognition of a peptide bound to MHC presented on APCs through the interaction with the TCR. T helper (Th) cells express CD4 and are important producers of cytokines that regulate the action of other immune cells, such as B cells and cytotoxic T cells. CD8+ T cells are able to directly kill cells through the release of effectors like perforins and granzymes. $CD8^+$ T cells recognize peptides derived from pathogens that are presented by MHC class I molecules (161).

The concept of different sets of Th cells, defined on the basis of their function, was introduced by Mosmann and Coffman in 1989 (171). Th1 cells are characterized by their production of cytokines interferon-γ (IFNγ), tumour necrosis factor α (TNFα) and interleukin 12 (IL12), among others. IFNγ is produced primarily by natural killer (NK) cells during innate responses in response to IL-12 and IL18 derived from phagocytes (161, 172, 173), and by $CD4^+$ T helper 1 (Th1) cells and $CD8^+$ cytotoxic T cells in response to antigens presented on MHC molecules (161). IFNγ is central to the defence against intracellular pathogens, by phagocyte activation and B cell production of opsonising antibodies that promote ingestion of microbes by phagocytes (161, 174). The differentiation of Th cells towards a Th1 profile is controlled by the T-box transcription factor T-bet (175-177) and signal transducer and activator of transcription 1 (STAT1) (178, 179). Th2 cells are characterized by the production of

interleukin IL4, IL5, and IL13, among other cytokines, and activate eosinophilic cells and stimulate B cells to secrete immunoglobulin E (IgE) (161). GATA-binding protein 3 (GATA3) (176, 180) and STAT6 (181, 182) are the main transcription factors governing the differentiation of Th cells towards Th2. Th17 cells use the transcription factor RORγ and produce the neutrophil activator interleukin 17A (IL-17A) together with interleukin 21 (IL-21) and IL-22 (183-186). Finally, T regulatory (Treg) cells, characterized by the transcription factor Forkhead box P3 (foxp3), have a crucial role in induction of self-tolerance against non-harmful antigens, and prevention of autoimmune diseases (187). Memory T cells can survive in a functionally quiescent state for many years (in mammals) after pathogen elimination, but have the ability to respond quickly to re-exposure of pathogens (161).

The term type 1 immunity encompasses a milieu skewed towards cytotoxic and other antimicrobial effector functions, including presence of natural killer cells, Th1, Th17 and $CD8^+$ cells and cytokines IFNγ and IL-12 [reviewed in (188)]. Type 2 immune responses are dominated by interleukins IL4, IL5, IL10 and transforming growth factor β (TGFβ) in addition to a number of different cells: Th2 cells, IgE secreting B-cells, alternatively activated macrophages, basophils, mast cells, eosinophils, mucus cells, epithelial cells and smooth muscle cells. Treg cells can also be found in type 2 environment [reviewed in (189)]. Wound healing and tissue repair are often governed by type 2 responses whereas type 1 constitute mechanisms effective at eradicating the pathogen.

Humoral antibody responses are initiated by the recognition of antigens by antigen specific B cells in mammals in the lymphoid follicles of spleen, lymph nodes and mucosal lymphoid tissues in mammals. Antibody responses to antigens can be T cell dependent or T cell independent, depending on the nature of the antigen. In mammals, the amount of antibody production is larger after the secondary encounter with the pathogen, and there are strong indications that this also occurs in fish. Activation of B cells results in a clonal expansion, and differentiation into plasma cells producing the effector antibody molecules. Antibodies are made up of heavy and light chains, with constant regions common to the same isotypes and variable regions in the heavy and light chains providing specificity. In humans, antibodies are classified on the basis of their heavy chains, and includes the immunoglobulin (Ig) isotypes

IgA, IgD, IgE, IgG, and IgM (161). Class-switching is found in higher vertebrates, with production of a different antibody isotypes after secondary encounter, whereas affinity maturation describes the production of antibodies with enhanced affinity to the antigen/epitope(s).

1.3.2.1 Immune responses in teleosts

Immune organs in teleosts

The two primary lymphoid organs in teleost fish are thymus and the bone marrow analogue head kidney (190-193). These tissues express *recombination activation gene 1* and *2* (194-196), *terminal deoxynucleotidyl transferase* (197) and *ikaros* (198) which are important for T and B cell development. Thymus is a paired T-cell rich organ in rainbow trout and Atlantic salmon located below the operculum and contains abundant T cells (190, 199, 200). Head kidney (pronephros) is aglomerular, the main site for haematopoiesis and contains mostly cells of lympho-myeloid origin, including IgM^{+} cells in trout (199) and Ig^{+} cells in Atlantic salmon (201) and possibly long lived antibody secreting plasma cells (193, 202, 203). MHC class II leukocytes with antigen-presenting cell characteristics are found in head kidney (204, 205) and homing of MHC class II^{+} cells from the abdominal cavity loaded with antigen are found here (205). The head kidney contains a sinusoidal system supported by a reticulo-endothelial stroma, which can trap particles from the blood stream (191). Furthermore, the more posterior parts of the kidney likely also have secondary immune organ functions (193). The posterior kidney houses B cells that can be activated into plasmablasts and plasma cells in trout, production of the latter can be stimulated by lipopolysaccharides (LPS) (193). Furthermore, the posterior trout kidney have a higher proportion of T cells than the anterior part, 1:1 and 1:2 of CD3ε^{+} and IgM^{+} cells, respectively (199). It has also been suggested that the melano-macrophage centres in the kidney may be a primitive analogue of lymph nodes in fish (206). They can retain antigens for long periods after administration (207).

The spleen is an important secondary immune organ, containing numerous IgM^{+} cells (199), likely important for the initial activation of B cells in addition to the posterior kidney (193). MHC class II^{+} cells home to the spleen from the abdominal cavity (205). The spleen also express highly diverse IgM, IgD and IgT repertoires after viral infection (208). The gill interbranchial lymphoid tissue contains abundant CD3^{+} cells, with fewer Ig^{+} cells and is likely a secondary immune organ (190). Fish do not have bone marrow, lymph nodes, germinal centres or B cell follicles (192, 209), thus it is not certain where stimulation of lymphocytes occurs

and effector sites are not well defined, but local activation and subsequent initiation of effector functions of lymphocytes are likely (209).

A mucosal associated lymphoid tissue (MALT) is associated with all mucosal surfaces in teleosts, the gut (GALT), the skin (SALT), the gill-associated lymphoid tissue (GIALT) and the

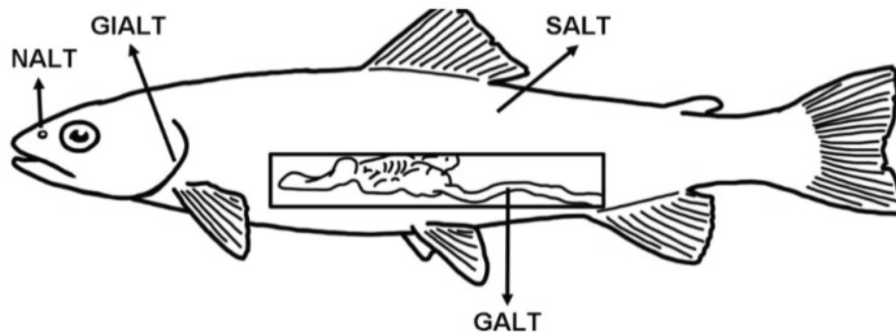

Fig. 6 An overview of the different MALTs found in teleost fish. Reused with permission from (209).

nasopharynx-associated lymphoid tissue (NALT) (209). Of these, the skin is the largest organ (Fig. 6). The SALT contains many of the important contributors of a functional adaptive immune response, including abundant $CD3\varepsilon^+$ cells in Atlantic salmon epidermis (200) and along the epidermal skin basal layer of rainbow trout (210). MHC class II^+ cells are also identified (56, 211), as well as $IL1\beta^+$ and $TNF\alpha^+$ cells in epidermis and dermis in coho, Atlantic salmon and sockeye salmon (56). In rainbow trout skin, abundant $CD3\varepsilon^+$ cells (199, 210) and $CD8\alpha^+$ cells (210) are reported, with 12 times more CD3ε cells than IgM^+ cells (199). In Atlantic salmon intestine, another MALT, the $CD3\varepsilon^+$ cells were found located in the epithelium between the enterocytes along the basal membrane (190, 200), only few cells were found to be $CD3\varepsilon^+$ in the *lamina propria* of healthy salmon (200). Rainbow trout gut harbour intraepithelial lymphocytes with high diversity of T cell transcripts; CD8, CD4, CD28, CD3ε, TCRζ, TCRγ, and TCRβ. These lymphocytes were responsive to viral infections by showing different CDR3 spectratype patterns (212). $CD8\alpha^+$ cells with high TCRγ transcript levels were found intra- and subepithelially in the gill and intestine of rainbow trout (213). IgT^+ B cells dominate at mucosal surfaces (214, 215). Furthermore, it has been suggested that teleost skin and gills show skewed responses, as there is a high constitutive expression of Th2 markers (216). After bacterial infection, this may change, as one study showed similar expression profiles of T-bet and IFNγ (217).

Innate teleost immunity

In fish, innate immune responses are particularly important, as they are considered temperature independent and more active at lower temperatures than adaptive immune activities (218). Furthermore, high affinity antibody titres take several weeks to develop (219). The inflammatory response in teleosts is biphasic, the first wave of cells approaching the area of infection are usually neutrophils, followed by monocytes and macrophages (220). Professional phagocytes in teleosts are monocyte/macrophages and neutrophils cells (221). Nevertheless, in recent years phagocytic properties have also been attributed to teleost B cells (222) and thrombocytes (223). The phagocytic properties of B cells, which may in fact surpass the neutrophils, have been shown in rainbow trout, Atlantic salmon and cod B cells (222, 224), including phagolysosome formation and intracellular killing of ingested microbes. Phagocytic B cells are present in blood, spleen, and head kidney and constitute the majority of all B cells (222). Based on this, it has been suggested that B cells are important in teleosts as a bridge between innate and adaptive immunity (225). Neutrophils and macrophages in fish appear to have similar properties as in mammals. This includes rapid infiltration at an inflammatory site (226), production of cytokines (227), ability to internalize latex beads (224), and ingestion of bacteria in the presence of opsonins (228). Neutrophils originate from the head kidney, while macrophages come from monocytes present in the blood. Monocytes are produced in head kidney and spleen (220). The specific genes for the NADPH oxidase complex that produces antimicrobial reactive oxygen derivates have been identified in rainbow trout macrophages (229). Similar to their mammalian counterparts, fish immune cells communicate and are modulated by cytokines. Classification of granulocytes in teleosts is under discussion. Mast cells are often named eosinophilic granular cells in fish because of their staining properties (220). Teleosts possess cells that are similar both structurally and functionally to mammalian mast cells in their digestive tract, urine bladder, skin and gills (230-234). In teleosts, metachrome staining of mast cells is observed using the same protocol recommended for identification of mammalian mast cells (232, 233, 235). The granules of mast cells contain vasoactive amines and proteases, which in mammals results in vascular and smooth muscle responses and inflammation (161). Acid phosphatase (236) and lysozyme (237) are found in eosinophilic granule cells/mast cells of rainbow trout and Atlantic salmon, respectively. The presence of histamine and staining properties of mast cells appear to vary substantially between fish species. Positive immunostaining with an antibody against

histamine was reported in mast cells of gilthead seabream, but not in rainbow trout, zebrafish, turbot and European eel (238). Heavy infiltrate of cells in gills of Atlantic salmon, which resembled mammalian eosinophils is also reported after amoebic gill disease. This may suggest importance in parasitic infections (239).

Natural killer cells and non-specific cytotoxic cells are identified in fish (240). Natural killer cells circulate in the blood, and can kill infected cells similarly to cytotoxic cells, but without need for prior activation (161). NK cells in fish are morphologically similar to mammalian counterparts, with conserved means of activation (240, 241).

Innate immunity during salmon lice infections

Signs of local and systemic activation of innate immune responses following experimental lice infection have been found in a range of salmonid studies (55, 56, 58-60). Given the presence of important components of immune system in salmon skin (209, 214), one would anticipate that the local immune defence in the host skin would be activated following *L. salmonis* infections and result in protection against infection. However, this is typically not the case in Atlantic salmon. Histopathological assessment of attachment and feeding sites in fin and gills of Atlantic salmon revealed minor host responses at the copepodid stage (60), similar observations through the juvenile stages in fin, gills and skin are also reported (60, 88). In comparison, resistant coho salmon show infiltration of neutrophils in the vicinity of the louse already at 1 day post infection (60). These immune responses are elicited subsequent to immune recognition of the parasitic invasion. Gene expression studies report early sensing of the louse in skin and systemically in spleen, liver and head kidney of salmonids (59, 124, 125), with activation of PRRs. One group of PRRs named lectins are important for recognizing carbohydrate domains on helminth surfaces (242). Tadiso *et al*. (125) found several lectins with early (1 dpi) induction in lice infected Atlantic salmon skin and spleen. Skugor *et al.* found also activation of lectins later into the infection in liver of Atlantic salmon (124). Furthermore, Tadiso *et al*. reported early induction of antimicrobial and acute phase proteins in Atlantic salmon spleen at 1 dpi corresponding to the copepodid stage. The authors described a change of character of the inflammatory gene expression changes after 5 dpi corresponding to the copepodid-chalimus moult, characterized by induction of genes related to tissue remodelling. However, responses did not result in protection, as fish harboured high counts (58.4 ± 9.48 lice per fish) at the end of the experiment (125). This study

also found down-regulation of antigen presenting molecules MHC class I in skin and head kidney during 1-5, and MHC class II in skin at 15 dpi. This could suggest absence of antigen presentation to T-cells in Atlantic salmon infected with *L. salmonis* (125). On this note, Braden *et al*. (56) found a high number of MHC class II$^+$, IL1β$^+$ and TNFα$^+$ cells at the adult lice attachment site in the lice resistant coho salmon.

Macrophages also responds differently during lice infections among salmonids. *In vitro*, significant suppression of respiratory-burst activity and phagocytic capacity in head kidney macrophages originating from infected Atlantic salmon at 14 and 21 dpi was found, which was not the case in macrophages from coho salmon and only at 21 dpi in rainbow trout macrophages (57).

The interferon (IFN) system is an important part of the vertebrate immune defence against viruses. Interferons are proteins, which have actions mainly by being secreted and activate antiviral systems in cells. The IFN system is also involved during parasitic infections, through their important role in shaping the subsequent T cell immune response (243-245). Suppression of IFN responses during infection with young stages of *L. salmonis* is reported among 3 salmonid species. Downregulation of a number of IFN-related genes, including receptors, intracellular signalling and regulatory factors in skin at 1-10 dpi in Atlantic salmon (125) and in chum salmon skin at 6 dpi and pink and chum head kidney at day 3, 6 and 9 after infection (59) are reported. In line with this, Krasnov *et al*. found an induction of interferon-related genes already at 3 dpi corresponding to 2-fold reduction in lice counts in a group of Atlantic salmon experimentally fed 17β-estradiol (70).

Adaptive teleost immunity

Teleost fish share many characteristics of T and B cell responses with mammals. In rainbow trout and Atlantic salmon, the identification of conserved molecular structures of TCRα (246, 247), TCRβ (248, 249) and TCRγ chains (250), and the great diversity in the TCR α/δ locus of Atlantic salmon (251) are suggesting a huge capacity for recognizing foreign antigens similarly to mammals. Co-stimulatory molecules CD4 (252, 253) and CD8αβ (254, 255) (Fig. 7) as well as numerous cytokines which sequences suggest highly similar T-helper (Th) Th1 and Th2 arms of immunity as mammals (188). For example, three IL4 related genes*, il-4/13a, il-4/13b1* and *il-4/13b2* were identified in both rainbow trout and Atlantic salmon. These three genes show different modes of expression after pathogen challenge in rainbow trout (256).

Upregulation of *il4/13* and *gata3* in rainbow trout epidermis (257) and kidney (258) are observed post parasite infection. Two paralogues of *ifnγ* are found in salmonids. Downregulation of *il1β* and *ifnγ* and upregulation of *il10* was reported after recombinant IL4 protein exposure in trout head kidney cells, suggesting opposing activities of Th1 and Th2 markers (258). Master regulators of Th1 and Th2; *t-bet* and *gata-3*, and their regulation following parasitic and bacterial diseases are described in rainbow trout (259) and Atlantic salmon (217, 260). *Gata3* expression is induced in spleen and head kidney of Atlantic salmon after *Aeromonas salmonicida* or LPS and β-glucan injections (260). Teleosts produce three antibody isotypes namely IgM, IgT (also called IgZ in some species) and IgD (261). IgM is the most abundant systemic antibody, whereas IgT is a specialized mucosal antibody in teleosts (214, 215, 262). IgT^+ B cells are the major B-cell subset in skin of rainbow trout (214). IgT^+ cells increase in number following parasitic infection at mucosal surfaces of trout (214, 215). The same studies showed that IgM responses were found confined to the serum (214) and that IgM^+ cells in *lamina propria* were not increasing in numbers following parasitic infection (215). There is indication of some degree of affinity maturation in fish (219, 263), but a good documentation for the underlying molecular events remains elusive in most fish species. Teleosts lack class switch recombination (CSR) despite the fact that they possess the gene coding for the enzyme responsible for this activity (264). Isolated enzyme from fish induces CSR in mouse B cells and fibroblasts (265, 266). Fish do not possess IgE, the Th2 specific antibody (209, 215).

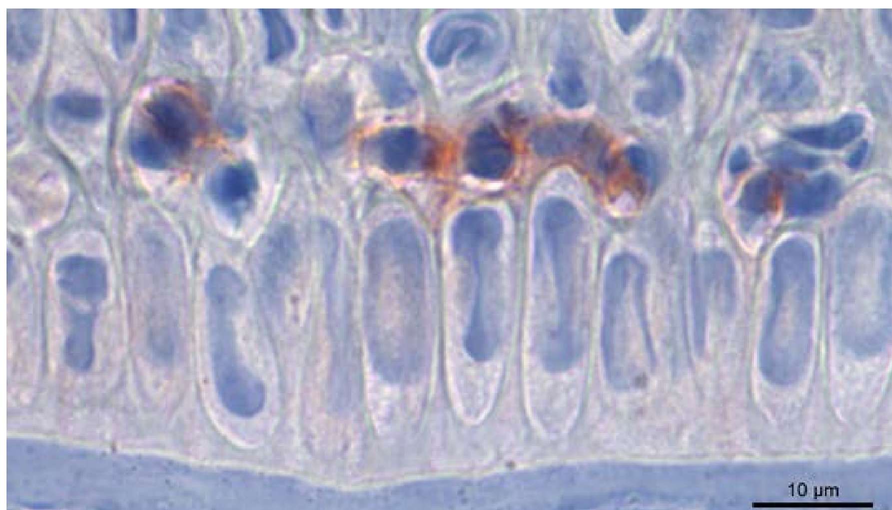

Fig. 7 $CD8\alpha^+$ cells (reddish colour) in Atlantic salmon epidermis. From paper I.

Adaptive immunity during salmon lice infections

The dynamic relationship between parasites and host immune responses is intriguing. The host may generate resistance mechanisms (kill the parasite), or tolerance mechanisms towards the parasite. The latter would entail "the ability to limit the health effects of parasites without preventing infection or controlling parasite replication" (267). During coevolution with their hosts, parasites have evolved mechanisms enabling them to overcome the host immune system and thus successfully finish their development into mature stages without killing and harming the host excessively. Excretions or secretions from the parasites may modulate the host responses to promote the survival and reproduction of the parasite. Many helminths and hematophagous insects induce highly polarized CD4 profiles and responses to parasites in mammalian studies are often illustrated within the Th1/Th2 paradigm (268). Major Th2 stimulating antigens may already be released from the egg stage of the parasite (269). Proteases secreted or excreted from helminths have been identified as Th2 inducers (270). Tick saliva also favor development of a Th2-type immune response in murine models, characterized by production of high levels of Th2 associated cytokines, and low levels of Th1 cytokines (271-274). In addition, tick saliva has been shown to push DCs to induce Th2-biased immune responses in vitro and in vivo (275, 276). Skugor *et al.* (124) suggested that attributes of the Th1/Th2 paradigm might explain differences in susceptibility to salmon lice among salmonids. Indeed, involvement of T cells during *L. salmonis* infections of salmonids has been shown at the transcriptional level in several studies (56, 124, 125). It is suggested that susceptible Atlantic salmon mount an impaired, Th2-like response (124). Th2 response may also provide a level of protection in resistant coho salmon (56). The latter study investigated responses to adult *L. salmonis*, and adaptive immune responses to parasites in teleosts can vary in nature related to the life stage of the pathogen (257). Differences in immune gene response to different stages of salmon lice have been shown for Atlantic salmon. A transcriptomic study showed contrasting responses to copepodid stage and after the transition to chalimus (125). At early timepoints in infected Atlantic salmon (1-5 dpi), activation of T-cells related transcripts in skin (TCRα and CD3ε) and head kidney (TCRα and CD3ε) was found. In contrast, by day 5-10, corresponding to copepodid to chalimus moult, the expression of these genes was suppressed (125). Dynamic regulations of a panel of immunoglobulin related transcripts were also observed in spleen, head kidney and skin in the same study.

Wound healing

The immune system of mammals may induce Th2 responses because of the sheer multicellularity of arthropods and helminths and their associated tissue damages (277). The mechanism driving Th2 immunity may thus be endogenous, i.e. through tissue damage and alarmins including IL33 (278), thymic stromal lymphopoietin (TLSP)(279) and DAMPs like uric acids (280) from damaged tissues and cells (281). Th2 immunity appear closely linked to wound repair processes. It is not surprising that blood feeding ectoparasites have evolved mechanisms to modulate aspects of the wound healing and angiogenesis, resulting in prolonged available time for their ingestion of the blood meal. This may also be the case during *L. salmonis* infections. The pathology of Atlantic salmon infected by high numbers of lice is characterised by gross skin lesions. Any break in the skin of fish must be rapidly repaired. Wound healing pathways in the host are commonly regulated transcriptionally during lice infections (70, 124, 125, 282). Wound healing consists of four events, including the initial haemostasis, inflammation to destroy pathogens, proliferation of various cell types and finally remodelling and maturation of the newly formed tissue, [as reviewed in (283)]. Homeostasis starts immediately after the wound is made, by vascular constriction and fibrin clot development. There is also a release of pro-inflammatory cytokines and growth factors. The inflammatory phase is necessary to clear off pathogens, where neutrophils play an important part. Macrophages, especially of the M2 type (281), are the major orchestrator of wound healing, by releasing cytokines and activating leukocytes and removing apoptotic cells, which pave the way for resolution of the inflammation. The proliferative phase is characterized by reepithelialisation over the provisional matrix within the wound. In the proliferative phase, fibroblasts and endothelial cells are prominent players, and growth of capillaries and granulation tissue at the tissue site occur. Fibroblasts produces collagens and other parts of the extracellular matrix. The final remodelling of the wound can last for years, to end in an architecture that is similar to the normal tissue. This also includes physical contraction by myofibroblasts (283). Wound healing in fish is temperature dependent (284, 285), but within optimal range can occur within hours. Covering of the wound gap in fish happens by migration of keratinocytes from the surrounding epithelium that meet from both sides, thus resulting in thinning of the adjacent epithelium (284, 286). Furthermore, fish keratinocytes can internalize bacteria and other particles, being an important contributor to defence against poten-

tially harmful pathogens (287). Central in the wound healing process is the metalloproteinases (MMPs), which are present in acute and chronic wounds as they participate in regulating extracellular matrix degradation and deposition essential for wound reepithelialisation (288). In normal tissue, MMPs are only expressed at basal levels, however their expression can be induced in keratinocytes, fibroblasts, endothelial cells and immune cells to a range of signals, including hormones and cytokines. Timed expression and activation of MMPs are essential, and specific MMPs are involved in the different stages of wound repair. Untimely and excess protease activity may lead to development of a non-healing wound (288).

Lice infections in skin are associated with MMPs upregulation at transcript level in skin close to the attachment and feeding sites of chalimus and older stages in Atlantic salmon (55, 70, 124, 125), and in pink salmon (55). Tadiso *et al*. reported induction of *mmp13* coinciding with the copepodid-chalimus moult (5-10 dpi) in skin, spleen and head kidney (125). Enhanced skin expression of *mmp9* and *mmp13* were also seen in fish exposed to cortisol and infected with chalimii alongside down-regulation of collagens and structural proteins (282). In Atlantic salmon, hepatic downregulation of *fibronectin*, a common substrate for MMPs, and increased activity of *mmp9* and *mmp13* in skin damaged by the chalimus and preadult stages were observed, which the authors suggested to be signs of a chronic wound development (124). The study concluded that Atlantic salmon responses to lice were consistent with the bias towards the Th2-like responses, but with impaired wound healing responses. Stress and cortisol release may very well worsen the consequences of lice infection on Atlantic salmon hosts. Cortisol injection and lice infection in Atlantic salmon affected many aspects of the wound healing cascade in a similar way. However, lice infected salmon showed a weak but consistent up-regulation of genes involved in tissue repair while cortisol had an opposite effect on these protective responses. Down-regulation of collagens and collagen modifying enzymes by cortisol could slow the healing, especially together with the up-regulation of MMPs that degrade ECM. Downregulation of motor proteins involved in wound contractions could also result in impaired repair of the skin (282). This could indicate a possible role of stress and cortisol in the lice induced pathology.

1.3.3 Systemic (physiological) responses to lice infection

Lice-induced skin damage includes direct effects related to the attachment and feeding activities, and indirect effects including stress, osmoregulatory disturbances and gill damage,

which may all lead to reduced growth and mortality (105, 143). Signs of visible stress after experimental infection with copepodids (6) and adults (56) are reported. Host responses to salmon lice infections are also dependent on the infection level. Many of the experimental laboratory studies use a large number of lice of the same stage to infect the fish, in order to tease out the host responses to infection. However, these responses may be different from those that would occur in the sea cage environment, where fish can be infected with several louse stages at the same time. On the other side, wild sea trout and Arctic charr can carry lice burdens, which have been found to cause osmoregulatory breakdown in experimental studies (6, 289). Marine teleosts live in a high salinity environment, and compensate for water loss by drinking substantial amounts of water. Lice induced skin damages can result in osmotic stress and in extreme cases breakdown of osmoregulation. Infection intensities above 29 lice per fish caused rapid increase in mortality in one study of experimentally infected Atlantic salmon post-smolt (initial weight 40 ± 6.3 g). This coincided with a significant increase in plasma chloride levels and 30 % reduction in haematocrit, which happened soon after the lice reach their pre-adult stage (6). Another study found high plasma chloride levels coinciding with mortality in experimental lice challenged Atlantic salmon (initial weight 60 ± 16 g) with an infection intensity at least 22 ± 8.1 preadults (46). Bjørn *et al.* found mortalities of 100 g sea trout rapidly increasing at the preadult stage with a mean infection intensity of 70 lice per fish (290).

Plasma cortisol levels have been used as indicators of stress in salmonids (291, 292). Lice density and cortisol level in blood of wild trout was found positively correlated (289). Cortisol concentration can be elevated in lice infected fish already at 7 days after experimental infection of Atlantic salmon (46), and may increase as the infection progresses (123). One study found positive correlation between plasma cortisol level and the individual infection intensity in experimentally infected Arctic charr (293). Only highly infected (178 ± 67) naïve Atlantic salmon showed significant increases of cortisol in another study (115). Cortisol levels may be affected by the louse stage, and elevated cortisol may result in reduced immune function in fish, shown as leukopenia *in vivo* (294), and reduced *in vitro* functions of leukocytes (295), and reduced innate immune response in head kidney (296). Coho salmon injected with cortisol showed higher lice counts than not injected controls over a 20 day study

period (297), outlining the role of cortisol as an important immunosuppressant for this species. In another study, Atlantic salmon injected with cortisol did not show increased susceptibility to lice, infection levels of injected and not injected fish were equal when the experiment was terminated (282).

Infection with lice could affect how fish respond to other diseases. Salmon lice may carry several fish pathogens, including the bacteria *Neoparamoeba perurans* (298), *Tenacibaculum maritimum, Pseudomonas fluorescens*, and *Vibrio spp* (299) and viruses such as salmonid anaemia virus (300-302) and salmonid alphavirus (303). Experimental challenge studies show that Atlantic salmon co-infected with the sea louse *Caligus rogercresseyi* and the bacteria *Piscirickettsia salmonis* show increased mortality (304). Atlantic salmon infected with *L. salmonis* and subsequently infected with the microsporidian *Loma salmonae* suffer higher burdens of the latter parasite (305). Furthermore, studies of Atlantic salmon suggest that sea lice could affect the disease resistance to *Neoparamoeba perurans* (298) and infectious salmon anemia virus (306, 307).

Regulation of iron as part of iron sequestration (withholding iron/heme from infectious agents) could be an important host defence strategy. Iron is an essential nutrient, which is used in heme. Furthermore, iron is a cofactor for production of antimicrobial oxygen radicals in leukocytes and iron levels can affect the Th1/Th2 shift of T helper cells (308). A number of parasites lack the pathway for heme biosynthesis, making heme acquisition from the host extremely important for survival (309). Controlling access to iron could be part of host defence against invading microbes and parasites. Haptoglobin, transferrin, hepcidin and other iron sequestration and regulating factors make the metal inaccessible to pathogens. Intracellular iron sequestration mechanisms may involve regulation of iron uptake pathways and activation of iron storage mechanisms (310), away from tissues that lice have access to (skin, blood and muscle). At the transcript level, induction of iron regulation both locally and systemically following exposure to *L. salmonis* has been found in experimental infection studies of Atlantic, pink, coho and chum salmon (56, 59, 70). Upregulation of the iron homeostasis regulator, *hepcidin-1*, which prevents export of iron from macrophages to the blood, together with other iron carriers in the circulation (*transferrin* and *transferrin-2* and *haptoglobin*) were found in skin (56) and head kidney (59) of resistant Pacific salmonids. The latter study showed also suppression of heme biosynthesis in pink salmon. Induction of transferrin

was also associated with protection against salmon lice in experimentally challenged Atlantic salmon fed sex hormones (70). Induction of iron restrictive mechanisms might be an important defence strategy in salmon against lice. Furthermore, lice possess complex iron regulatory mechanisms. Iron regulatory proteins, known to play crucial roles in handling excess iron that can be toxic to the parasite, was recently found in *L. salmonis* (311).

1.4. Biological approaches for salmon lice control

Integrated pest management (IPM) strategies was originally developed in agriculture to manage the plant pests and a number of definitions can be found (312, 313). A consensus of definition was proposed by Kogan in 1998, stating that "IPM is a decision support system for the selection and use of pest control tactics, singly or harmoniously coordinated into a management strategy, based on cost/benefit analyses that take into account the interests of and impacts on producers, society, and the environment" (313). IPM for salmon lice was introduced by Mordue and Pike in 2002 (314). This approach for management of salmon lice infections encompasses the use of several chemical and non-chemical tools to solve the lice problem, and requires a comprehensive understanding of the host, parasite and the environment to tailor an effective plan for each site and area. Non-chemical alternatives for salmon lice management are also on the market, but their individual effectiveness in reducing lice levels on farm are generally considered inferior to chemical interventions. Biological control of salmon lice by the use of co-culture of cleaner fish and salmon has gained attention as an environmental friendly and cost-effective option for salmon lice management since the late 1980s (315). Wild caught wrasse as lice cleaners were already tested in laboratory trials in 1988 (316), followed by promising experiments in sea cages (317, 318). The species of wrasse used include; goldsinny wrasse (*Ctenolabrus rupestris*), corkwing wrasse (*Symphodus melops*) and Ballan wrasse (*Labrus bergylta*) (3, 319). In addition the lumpsucker (*Cyclopterus lumpus*) is also used (3). As the commercial fisheries are insufficient to meet the amount of cleaner fish needed in salmon farms, cultivation of Ballan wrasse and lumpsucker has developed over the past decade (320) (3). In 2015, 10 million lumpsuckers and 400 000 - 500 000 individuals of Ballan wrasse were produced in Norway (3). Cleaner fish may be efficient at removing lice. A recent study found that farmed Ballan wrasse at a 5 % sea pen stocking rate resulted in reduction in mean sea-louse count from ∼12 to 0.4 lice per salmon (321), and no differences in delousing efficiencies of the farmed and wild Ballan

wrasse were found (319). However, criticisms have been raised against the wrasse industry. This is related to the catching methods of the wild wrasse, which frequently involves bycatch of other fish, lobsters and otters, but also decreased welfare of the wrasse kept in the sea cages (322). This includes observations of high mortality, reduced life expectancy and potential risk of disease transfer between the wrasse and farmed salmon (323, 324). Wrasse are less effective when the salmon grows bigger (27).

1.4.1 Vaccines against lice

A present, efforts towards sea lice vaccine development are initiated by several research groups worldwide. Vaccines could be a cost-effective approach for controlling sea lice. In contrast to chemotherapeutants, vaccines can offer a prolonged duration of action and no withdrawal time (325). On the other side, the complex life cycle of parasites, and the magnitude of antigens expressed by each stage makes vaccine production challenging (326). Furthermore, immunity may for most ectoparasites only lead to partial resistance (327). The best example of a commercially and effective vaccine against an ectoparasite was developed against the tropical cattle tick *Boophilus microplus* (328). This vaccine was made against an antigen in the gut of the tick. Several research groups have attempted to make a vaccine based on salmon louse gut antigens. The successful vaccine development is hampered by the fact that the louse gut may degrade the vaccine antibodies. Furthermore, salmon lice are not specialized blood feeders, and it is uncertain how important blood is for the louse to complete its lifecycle. Compared to specialized hematophagous parasites as ticks, lice likely ingest little amount of host antibody (325). One of the earliest salmon lice vaccination studies used fractions from *L. salmonis* extracts to make rat-antisera that was later used for immunohistochemistry. The fractions that bound to the gut lining of the louse was suggested to be good vaccine candidates (329). Antibodies made from louse extracts that could bind to lice tissue sections, were also identified in a parallel study by another research group (330). Grayson *et al*. also found that rat antibodies made from purified extractions of lice could bind to gut epithelium, but subsequent challenge trials showed no differences between the immunized group to the control group. However, there was a significant reduction in the abundance of gravid female lice on the immunized fish (331). These results should not be overestimated, due to the lack of group replication and estimates of tank variability (325). A number of potential antigens for salmon lice vaccines have been patented (332-

335). Recently, a novel antigen candidate, akirin, was identified in *C. rogercresseyi* (336). Akirins are considered good vaccine candidates for reduction of tick, mosquitoes, sand flies and mite infections in mammals (337). Another vaccine formulation was launched in Chile in 2015, said to result in 73 % reduction in *C. rogercresseyi* lice number. In addition to the lice antigen, a potent immunogenic protein, the formulation includes “mucus stimulating factor”, which was said to stimulate the production and improve the mucus quality in the fish skin (338).

1.4.2 Selective breeding for increased resistance against lice

Another promising part of the IPM toolbox for salmon lice management is the use of selective breeding for increased resistance to lice. Resistance can here be defined as the ability to limit parasite burden (339, 340). Breeding for disease resistant fish was described already in 1926, where trout survivors from natural outbreaks of furunculosis were used to breed new generations more resistant to disease than previous generations (341). Inclusion of traits of resistance to furunculosis, infectious salmon anaemia (ISA) and infectious pancreatic necrosis (IPN) in breeding programs for Atlantic salmon in Norway originated in the 1990s (342, 343).

During challenge trials with salmon lice and Atlantic salmon, a skewed distribution of lice is commonly observed, where a few fish harbours many lice but most fish have lower lice burden (66, 67, 344). The continuous variation in host abundance of lice suggest that resistance of susceptibility is a quantitative trait, influenced by a large number of genes, and also influenced by environmental factors (345). Phenotypes, in this case lice burden, can thus be assigned the sum of genetic and environmental effects:

Phenotype (P) = Genotype (G) + Environment (E)

Variance (P) = Variance (G) + Variance (E)

In breeding programs, where controlled mating will occur, it will be possible to calculate the variances. The total genetic value of an individual can be separated into the following parts:

G = A + D + I

A is the additive genetic variance. The additive variance is important for selection, and can be defined as variation due to the sum of individual gene effects. D is variance related to interactions between alleles within loci; dominance effects, and I is the epistatic value (the effects of interactions between loci) (345). The level of additive variance is important for the choice of breeding approach.

Heritability is important for quantitative genetics. It can be used to estimate breeding values and responses of selection. In a broad sense, heritably (H^2) assigned to all the genetic contributions of the phenotypic variance, and is calculated by this formula:

$$H^2 = \frac{\mathrm{Var}(G)}{\mathrm{Var}(P)}$$

Non-additive genetic variance (D, I) is not transmitted to the offspring, thus the broad sense heritability will not give correct breeding values (345). In selective breeding, the additive variance due to the parent-offspring resemblance forms the narrow-sense heritability (h^2) defined as:

$$h^2 = \frac{\mathrm{Var}(A)}{\mathrm{Var}(P)}$$

Heritability for traits in aquatic animals is typically in the range of 0.1-0.4. The heritability estimates can only be used for the population that provided the estimate. However, general application of heritability estimates can be more reliable if they originate from a large dataset (345). Heritability estimates are used to estimate the breeding value, A_i of trait X of an animal:

$A_i = h^2 (X_i$ – population average (X mean))

The larger number of animals that contributes to the phenotypic average of the family, the better estimate of the additive genotype of the family will be made. This is because environmental effects will approach zero when the number of individuals in each family is increased. Application of mixed models are commonly used to predict breeding values.

Best Linear Unbiased prediction (BLUP) models, also named animal models, are commonly used:

$$y = Xb + Za + e$$

Where y is the phenotype vector, b is the vector of fixed effects with design matrix X, a is the vector on random effects with design matrix Z and e is a vector of random residual effects (67).

Challenge tests are considered more accurate and reliable than natural infections in the field for selecting the breeding candidates, as there is more control over the environment and course of infection (345). The importance of a consistent challenge methodology for studying parasitic resistance was outlined in a study with conflicting results; a dominance for susceptibility was found with continuous parasite exposure, but a dominance for resistance found with pulse exposure (346). Furthermore, quantitative traits may be associated with one another, either because they are controlled by similar genes or because they are affected by same milieu factors (345). For instance, positive genetic correlation between number of lice and fish weight has been found in several studies (65, 66, 347).

In fish farming, selective breeding is either performed on the individual or family level, depending on the trait. If siblings are not available, then selection would be based on individuals showing the trait of interest. Progeny testing of male candidates is often used in livestock, but in salmon aquaculture, the extremely high fecundity of salmon makes organized breeding programs based on sibtesting a good alternative. The accuracy of breeding value estimation is high since the family sizes in aquatic species are often large (348). Sibtesting is however limited by the fact that candidates are selected based on the parent mean (67). Furthermore, selection of candidates for increased disease resistance based on challenge or field trials is hampered as the challenged fish cannot be used as breeding candidates themselves. However this may not be the case with salmon lice as the fish can be deloused and evaluated as breeding candidates themselves (66). In selective breeding trials for increased lice resistance, selection strategies have been based on family selection, and not individual selection. Family selection is a strategy in which breeding candidates are graded based on information from their full and half-siblings; in this case, the information would be lice counts. Thus, selection is limited to selection among untested individuals based on results

from tested relatives, mostly full- and half-sibs (348). This means that the breeding companies must choose individuals from high-resistant families, rather than individuals across families, and there is no information about the within-family variation (349). Traits with low heritability, especially disease resistance, is highly suited for family selection (348).

Selective breeding for increased resistance to parasites has been studied for internal parasites in humans and livestock (350-355), and increased parasite resistance has been used as a criteria in selection breeding programmes in ruminants for decades with success, and also resulted in lower use of antihelminthics (356, 357). In ruminants, faecal egg count has been used as the selection trait, showing a moderate heritability (350, 358, 359), and selective breeding has resulted in substantial reductions in parasite egg shed (360). Calculated heritabilities for bacterial and viral diseases in Atlantic salmon are generally moderate to high, ranging from 0.1 to 0.4 depending on the pathogen (345). Species of salmonids differ in their susceptibility to salmon lice (57, 59, 60), and variation also occurs within stocks of brown trout (*Salmo trutta*) (361) and wild and farmed Atlantic salmon stocks (362). Furthermore, there is commonly a large variation in lice numbers within a population of Atlantic salmon after experimental challenge (65, 66, 362), in natural settings (65, 99), and between Atlantic salmon families (65, 363). Heritability calculations of sea lice resistance have this far been based on lice counts and information of pedigree, and the magnitude of the heritability observed depends on the stage of louse that was counted, and whether the infection happened in natural setting or experimental setting. Gharbi *et al.* found the number of lice and ranking of families similar at 7 and 17 dpi, suggesting that resistance/susceptibility to lice is decided early in the infection (344). Lhorente *et al.* calculated heritability for resistance against *C. rogercresseyi* and found low (0.03–0.06) to medium (0.22–0.34) heritabilities for the mobile and sessile stages of the parasite, respectively (364). Heritability to *L. salmonis* was low-moderate during natural infection of motile [0.02 ± 0.02] and sessile [0.12 ± 0.02] stages, respectively, and also for total number of lice [0.14 ± 0.02] (65). Heritability for sessile lice was substantially higher [0.33 ± 0.05] in a controlled infection trial by Gjerde *et al.* (66) and for the fish that were subjected to a controlled challenge test in the Kolstad *et al*. study (65) [0.26 ± 0.07] of total number of lice. Gjerde *et al.* (66) stated that “the level of observed heritability together with the large variance in phenotype suggests ample additive genetic

variability, and thus a good potential for increasing the resistance through selective breeding", supported by (344, 365, 366).

These studies calculated heritabilities based on lice counts, which certainly has its weaknesses. Counting adult lice may be inaccurate due to the host switch behaviour of adult lice (99). Furthermore, lice abundance at a given time point might not reflect the total number of lice that have been found on the fish throughout the sea period. Studies also show that lice abundance on salmon infected with salmon lice for the first time is a poor predictor of the infection level the second time (65, 367).

The use of marker assisted selection (MAS) for Quantitative trait loci (QTLs) has been exploited for several viral and bacterial diseases in teleosts (368-370), particularly successful for the IPN QTL in Atlantic salmon (371). Furthermore, a QTL for increased lice susceptibility (so-called "lice samplers") has been found, and roe without this QTL is in market from 2016 (372). A promising alternative for increasing genetic resistance to lice could also be the use of genomic selection, which exploits several genome-wide markers in the analysis, and does not require an prior knowledge of the QTL (373). Using an combined approach of disease challenge tests to find the best families followed by gene expression profiling to select the best individuals within the family could be a promising approach in Atlantic salmon (374).

1.4.3 Anti-lice functional feeds

Feed is the single highest expense in modern fish farming. Scientific data gathered over the past thirty years indicates that dietary nutrients as well as additives could stimulate the immune system of fish and help to reduce symptoms, pathology and mortality following infectious diseases of fish, in addition to cover the basic nutritional requirements for the immune system. Feeds enriched in bioactive compounds can be incorporated into feeds and used over a short period at particularly vulnerable stages of fish development or when it is anticipated that imminent infections may occur. Functional feeds (FF), defined as feed containing components that "aid specific bodily functions in addition to being nutritious" (375) is a growing market, especially in the management against infectious diseases in aquaculture. The most common additives used in aquaculture diets are probiotics, prebiotics, immunostimulants, vitamins and nucleotides (376), several of which have been investigated in the management against salmon lice.

1.4.3.1 Potential of phytochemicals found in Brassicas

A novel and promising approach in the battle against lice entails the use of in-feed phytochemicals. Phytochemicals of interest are secondary plant metabolites named glucosinolates (Gls) derived from plants in the family Brassicaceae. The basic structure of Gls consists of a β-thioglucose moiety, a sulfonated oxime moiety, and a variable side chain (377). Gls and their breakdown products have a range of *in vivo* and *in vitro* effects shown in invertebrates and vertebrate studies. Gls are potent feeding stimulants for numerous insects specialized on Brassica plants, but Gls and their derivatives are also used by the plant to repel or deter generalist herbivores (378). In an interesting study by Newman *et al*., old watercress was preferred by the snails and insects tested in the study compared to new watercress, which was linked to the much higher glucosinolate content in the younger plants (379). Gls and breakdown products have potential as natural pesticides against nematodes, fungi and bacteria (380-382), and control of weeds (383, 384). Variations in the side chains can assign the Gls into three main groups; aliphatic (50 %), aromatic (10 %) or others (30 %) (377). Gls have intrinsic defensive properties against herbivores (385), but most actions are enhanced with the hydrolysis products mediated by the enzyme myrosinase, stored in myrosinase cells in plant organs (386, 387). The enzymatic reactions happen with tissue damage, but outcome is depending on which Gls are present, the pH, metals and other proteins in the cells. The reaction can also be mediated by the gut microbiota in humans, the reaction is inhibited by antibiotic treatment (388). Aliphatic Gls yield isothiocyanathes (ITC) at neutral pH (378), and it is the derivative of Gls mostly associated with biological functions (389) including induction of mortality and stress in insects (390-392) and *in vitro* antibacterial (393, 394) and antifungal (381, 395, 396) properties. In mammals and fish, available literature describes the advantages and drawbacks of exposure to Gls and their derivatives. Thiocyanate anions compete with iodine for active transport across the cell membrane (397). Reduced feed intake and growth with intake of glucosinolates is reported in a range of birds, monogastric and ruminant animals (reviewed in (398)), related to the bitter taste and acrid smell (399, 400) and goitrogenic effects due to iodine competition in pigs (401) and rats (402). Due to the good amino acid profile in rapeseed meal (RM), incorporation into fish feed has been of interest, but proven difficult due to the high fibre levels and Gls content. In Burel *et al*., decreased growth in juvenile rainbow trout fed Gls was observed already at 24 days after start feeding, resulting in a 30 % lower bodyweight at 58 days of feeding. This was likely related

to a dysregulation of the thyroid hormones already found after 14 days in trout fed Gls, which could be reversed by dietary supplementation of external T3 or iodine (403). In another study of rainbow trout, reduced growth after 3 weeks and thyroid disturbances after 9 weeks of feeding was found in fish fed Gls, shown by lower plasma levels of T3 and T4 and a hyperactivity of the thyroid follicles (404). In Glencross *et al*., levels of T3 and T4 in blood of red seabream *(Pagrus auratus)* fed Gls at for three weeks did not change (405).

A number of positive biological effects of Gls exposure in vertebrates are also reported in the literature, mostly associated with ITCs. The cytoprotective effects of ITCs are mediated through their ability to inhibit phase I and activate phase II detoxification (406). They are thus considered to be indirect antioxidants, since they do not inhibit activity of free radicals by themselves, but through modulation of phase I and II enzymes. Phase I enzymes include the cytochrome p450 enzymes, that increases reactivity of fat-soluble substances, which may result in their increased toxicity. Phase II enzymes include glutathione-S-transferase, aldehyde reductase, S-methyl transferase and N-acetyltransferase that increase the water solubility and facilitate excretions of the metabolites out of the body (406). Sulphoraphane (SFN), an ITC, is suggested to be one of the most potent naturally occurring phase II enzyme inducers (407, 408). SFN works through the Kelch-like ECH-associated protein 1 (Keap1) – Nuclear factor E2-related factor 2 (Nrf2)–antioxidant response element (ARE) pathway, resulting in increased ARE transcription (409) (Fig. 8). The cytoprotective proteins share a common transcriptional way of modulation through the Keap1–Nrf2–ARE pathway (410). Under basal conditions, dimeric Keap1 (Fig. 8a, blue) serves as the ubiquitin ligase substrate adaptor that presents Nrf2 (purple) for ubiquitination. The polyubiquinated Nrf2 (the ubiquitin molecules shown as intermixed dark red and orange) enters the proteasome and results in degraded purple Nrf2 fragments (Fig. 8a, purple). Fig. 8b shows the situation under induced conditions, where SFN bind and chemically modify the cysteine residues (red sticks) of Keap1 (blue), resulting in change of conformity and loss of ability to target Nrf2 for ubiquitination. Accumulation of Nrf2 commences and heterodimerization of Maf transcription factor (green) occurs, resulting in the complex binding to the antioxidant response element (ARE) in the promoter region of cytoprotective genes, stimulating their transcription.

There is increasing evidence that ITCs, due to their ability to modify the Keap1–Nrf2–ARE pathway, can inhibit the development of a number of chronic diseases through chemoprevention (411). Chemoprevention is defined as the use of natural or synthetic agents able to reverse, inhibit, or prevent the development of a chronic–degenerative disease, where chronic inflammation is a key player. Loss of Nrf2 signalling results in enhanced susceptibility to oxidative and electrophilic stress and to inflammatory tissue injuries in humans (412). This can be reversed by SFN stimuli shown in human and murine *in vivo* studies for a range of disorders (reviewed in (411)), including asthma (413), cardiovascular diseases (414, 415), cancer development (416-418), diabetes (419), and neurodegenerative diseases (420, 421). In skin, evidences of Nrf2 role in controlling wound healing was found in (422). Nrf2 can be activated by UV radiation of dermal fibroblasts (423), and result in cell rescue especially at low DNA damage levels (424). Another study also showed that SFN restored the age and Nrf2-related decline of Th1 immunity in skin of old mice through a proposed restoration of redox equilibrium (425).

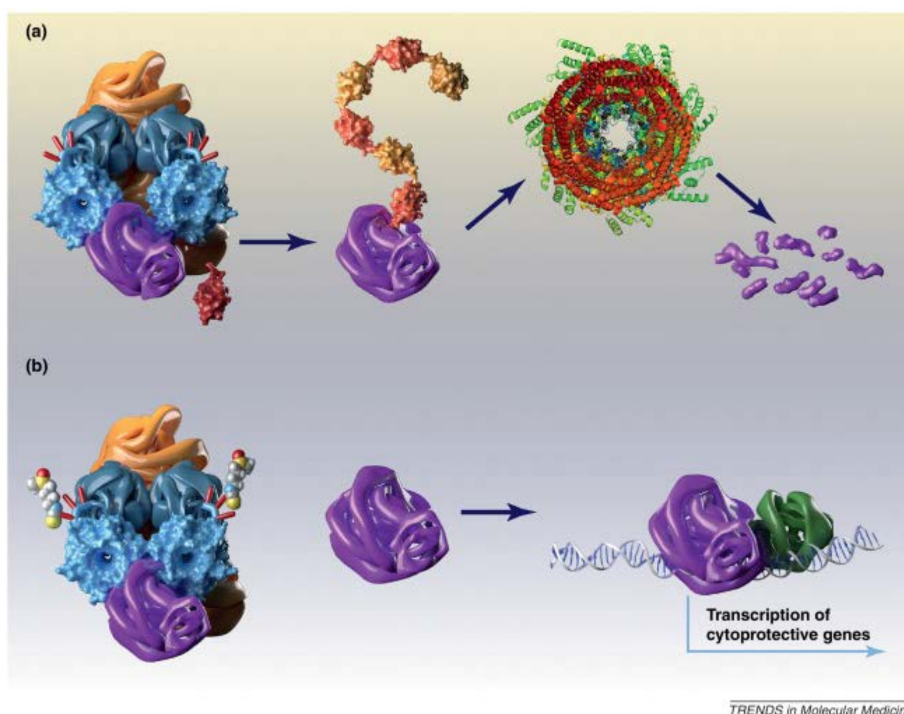

Fig. 8 Model of the Keap1–Nrf2–ARE pathway. (a) basal conditions.
(b) induced conditions. Used with permission (387).

1.4.3.2 Immunostimulants (PAMPs)

Due to the many observations linking the magnitude and characteristics of the immune response to reduction in lice counts, several studies have been addressing the use of in-feed immunostimulants that contains pathogen-associated molecular patterns (PAMPs) to combat lice. The vertebrate host immune system will consider these structures foreign because they contain molecular and structural characteristics commonly found in microbes, but very rarely in vertebrates (426, 427). One such is β-glucans, glucose polymers, especially glucans containing a backbone of b(1,3)-linked b-D-glucopyranosyl units with b(1,6)-linked side chains of varying distribution and length. These are commonly found in nature as part of walls of yeast, plants, bacteria and fungi (428, 429) and known to elicit biological responses in vertebrates (430), where receptors for beta-glucans are widely expressed (431). β-glucans are reported to modulate innate immune responses and complement in fish (429, 432). In one trial, lower levels of *L. salmonis* during natural infection was observed in Atlantic salmon fed yeast derived β-glucan (433). On the other side, Covello *et al*. found increased *L. salmonis* abundance after experimental salmon lice infection in Atlantic salmon fed another source of β-glucan. The same study showed reduced lice levels in Atlantic fed brewer's yeast (a yeast ß-(1,3)/(1,6)-D-glucan product) (434). Another study found little reduction in lice numbers using commercial yeast extracts (435).

Another example of a potential PAMP containing in-feed substance that can be used to enhance fish innate immune defences is unmethylated DNA, which contains cytosine- phosphodiester-guanosine oligodeoxynucleotide motifs (CpG ODN) (436). Bacterial unmethylated DNA is commonly found in prokaryotic DNA, but not in eukaryotic, and thus acts as a foreign danger signal through TLR9 (437, 438). The CpG ODN 1668 has been tested against salmon lice. Covello *et al.* first addressed feeding 20mg/kg feed for > 5 weeks to Atlantic salmon exposed to multiple salmon lice experimental infections. Results showed a trend of reduced lice counts at 10 days, 17 and 37 dpi, but only the latter was significant. The fish fed CpG ODN also had fewer skin ulcers and higher inflammation scores on histology (434). A similar study addressed the effects of multiple exposures of *L. salmonis* in Atlantic salmon fed 10 mg/kg of CpG ODN. It was found that re-exposed fish showed increased regulation of cytokines *il1β* and *il12β*; and suppression of *mmp9* in skin and spleen. At 7 dpi, there was a significant 46 % reduction in lice numbers in fish fed CpG ODN. This was in comparison to

fish fed standard feed and exposed to lice for the first time. This trend of reduced lice levels was also maintained later into the infection but was not significantly different. No differences were found at the attachment site by histological evaluation (439). A possible combinatorial effect of CpG ODN and the anti-lice chemical emamectin benzoate has also been tested. A trend of reduced lice counts, although not significant was observed, which authors speculated was due to a suboptimal dosing of the compound (435).

2. Objectives

The overall objective of the present study was to investigate host responses to lice infections in Atlantic salmon with an aim to obtain a better understanding of which host mechanisms are involved in limiting the infection success. Then to modulate and strengthen favourable responses by selective breeding and nutritional components.

The specific objectives were:

- Understand interactions between salmon louse and Atlantic salmon with a particular focus on skin histology and immune responses.
- Study skin histology and gene expression of immune candidates of protection in genetically different stocks of Atlantic salmon, bred either for increased resistance or susceptibility to lice.
- Study to what extent feed components can modulate immune responses and impact on the outcome of lice infection.
- Study molecular underpinnings of protection against lice conferred by a functional feed; by assessing transcriptomic responses in affected tissues.

3. Summary of Papers

Paper I

This study investigated the early skin responses in Atlantic salmon to lice infection. The time points selected for tissue sampling were 4 dpi and 8 dpi, to capture the host reactions to copepodid and chalimus stages of *L. salmonis*, respectively. Skin from two sites were sampled, including tissue from the occipital part of the head and behind the dorsal fin, which are two preferred locations for louse settlement and feeding activities, but differ in their tissue constitution as skin from head lacks scales. Skin behind dorsal fin is covered by scales. Skin tissues sampled from two locations were analysed by qPCR of 32 genes. Furthermore, skin from head was also subjected to immunohistochemistry using antibodies raised against CD8α, Mx and MHC class II. Samples from not-infected fish were also included. By comparing the gene transcript level between these sites of skin in not infected fish, basal/constitutive differences were found for many immune-related genes between the two skin sites. *Mannose binding protein C* was over 100 fold higher expressed in scaled skin. In infected fish at 4 dpi, the majority of genes at both skin sites showed lower values than in the non-infected control, which could be linked to lice mediated immunosuppression. A temporal increase from 4 to 8 dpi was evidenced for most transcripts, including cytokines of Th1, Th17 and Th2 pathways. Immunohistochemistry revealed that MHC class II$^+$ cells were evenly distributed across the epidermis, including keratinocytes on the top layer close to the surface. Mx$^+$ and CD8α$^+$ cells were found close to *stratum basale*, and there was a small but significant, increase in numbers of CD8α$^+$ cells in response to infection. This revealed a minor activation of T-cell defences in skin of Atlantic salmon during infection with young stages of *L. salmonis.*

Paper II

Atlantic salmon is susceptible to *L. salmonis,* but there is variation in susceptibility within the species can be exploited in selective breeding programs for increased lice resistance. This study used lice counts from 3000 siblings from 150 families of Atlantic salmon to rank the families into high resistant (HR) and low resistant (LR). By subdividing the groups by lice number: HR < 10 and HR > 10, and LR < 10 and LR > 10, an understanding of the effect of lice burden *per se* apart from family background could be made. Skin was sampled from behind the dorsal fin (nearby lice attachment) from the ten extreme families (HR or LR) and analysed

by qPCR for 32 candidate genes, including genes involved in T helper cell (Th) mediated immune responses, found to be regulated during lice infections in previous studies. In addition, morphometric analysis of the epidermis was performed. The use of multivariate statistics enabled us to explain the relationship between the genes, lice count and the fish groups. Large variation in lice counts within resistant and susceptible families was found. In the fish selected for analyses in paper II, HR fish had 36 % reduction in lice numbers compared to LR fish. Both genetic background and the number of attached parasites influenced expression patterns. The impact of immunosuppression by lice was evidenced in LR fish. The strongest down-regulation of most genes was seen in LR > 10, with an inverse correlation between the LR fish and IFN and Th1 markers. On the other side, HR fish were better able at resisting suppression of expression of both Th1 and Th2 related genes. Morphometry showed that LR fish had thicker epidermis and larger mucus cell size compared to infected HR fish, although hardly significant ($P = 0.08$). The findings suggest that a reduction in lice number is possible with the use of selective breeding. The ability to resist lice infection depends on the ability to avoid lice mediated immunosuppression.

Paper III

This study investigated the use of phytochemicals named glucosinolates (Gls) in reducing lice infection levels. Gls are used in nature as protection against herbivores by plants in the Brassicaceae family. A range of studies in animal models and humans report health-promoting effects following Gls ingestion and exposure, related to their antioxidant and detoxifying properties. The use of Gls enriched functional feeds to manage lice infections has not been studied previously. This study aimed at feeding Atlantic salmon two different doses of Gls-enriched feeds. The effects of feeding high dose of Gls before the infection, and of high and low doses five weeks into the infection were studied by using 15 k oligonucleotide microarray and qPCR. For comparison, infected and not infected groups of fish fed control diet were also included in the study. Lice (preadults and adults) were counted on 180 fish (60 fish per dietary group). In the group of fish fed low dose of Gls, a 25 % reduction ($P < 0.05$) in lice counts was found compared to fish fed control feed. A 17 % ($P < 0.05$) reduction was found in the group fed high dose of Gls.

Microarray analysis revealed induction of over 66 interferon related genes prior to lice infection in the high dose group. 5 weeks into the infection, several of these genes were also

upregulated in the infected fish fed high dose of Gls. In both infected Gls exposed groups, induction of antimicrobial and acute phase proteins, T cell effectors, complement, lectins and iron homeostasis regulators were discovered. The study also found suppression of genes encoding lipid metabolism, tissue differentiation regulators and ECM components in Gls fed fish. In contrast, genes involved in muscle contraction, lipid and glucose metabolism were found more highly expressed in the skin of infected control fish. It was discussed in the paper whether the regulation of iron, lipids and sugar might interfere with the host recognition, attachment process and development of the parasite. Overall, findings in paper III suggest that reduced lice counts in Gls fed groups of fish were due to activation of Th1 genes and several antimicrobial protein related genes.

Paper IV

In paper III, a reduction of *L. salmonis* counts was accomplished in groups of fish fed Gls. However, wider application of Gls demands for a thorough knowledge on the positive and negative effects of Gls exposure. Paper IV included analyses of data from three trials, where fish were fed a high level of Gls (Trial I), a high and low level of Gls and infected with *L. salmonis* (Trial II), and low levels of Gls and infected with *L. salmonis* (Trial III). In all trials, fish groups fed control feed were also included. Fish weights were significantly reduced with Gls exposure in Trial II, and in all trials, a lowering effect on liver steatosis was found with increasing inclusion level of Gls. Microarray analyses supported these findings, by showing signs of reduced cellular proliferation in liver and muscle. Furthermore, in distal kidney, activation of anti-fibrotic responses could be a result of toxic effect from Gls overexposure. On the other side, enzymatic plasma profiling in Trial I and II could not evidence clear signs of organ damage. Prevalent activation of phase-2 detoxification and iron regulatory genes occurred in all three tissues by high dose of Gls. This may pose beneficiary for the fish during lice infection, as lice depend on the host for iron/heme. Dietary Gls have multiple systemic effects in Atlantic salmon, and their successful application in feed requires a thorough understanding of how beneficiary effects may be promoted without posing adverse risks on the health of the fish.

4. Methodology

4.1. Fish experiments and study designs

The impact of *L. salmonis* on Atlantic salmon is best studied through *in vivo* experiments, in which experimental studies are performed to closely mimic the natural conditions.

In all papers, fish were infected experimentally with *L. salmonis*, and lice were staged and counted on anaesthetized fish. However, in paper I, III and IV, the experimental infection and feed studies were performed indoors, in 500 L circular flow-through tanks, by lowering the water height and distributing copepodids into the water. Lice were counted when majority of lice reached preadult and adult stages. All trials in paper I, III and IV were performed at Ewos Innovation's Test Facility in Dirdal, Norway, an experienced experimental facility running 3-4 large-scale experimental lice and feed studies every year. Running challenges in a controlled in-door facility where milieu parameters such as water temperature, salinity and oxygen satiation are continuously monitored and controlled, allows for safer comparison of results between different trials. Furthermore, clinical signs of disease will be discovered as the fish in the tanks are observed on a daily basis. To exclude possible in-between tank variation, all fish fed experimental feed originated from three tank replicates and efforts to include fish from all tanks in the subsequent analyses were made. In paper II on the other hand, fish were infected in sea net-cages, using closed tarpaulins and lice were staged and counted at the chalimus II stage following procedures that were developed previously for a similar experiment (66). This allowed for easier comparison to previous findings. Lice were counted at the chalimus stage since sessile are less likely to fall off the fish when being caught and anaesthesia of several fish at once in a small container can be performed directly after fish are caught from the net cage. This makes the sampling more efficient, and reduces stress for the fish, since the sea net-cages and the lab facilities were situated apart from one another in this challenge facility. The fish were individually pit-tagged, to allow for efficient and accurate identification, since fish from all groups were cohabitated. In paper II, there was a difference in the number of fish in each group and variation in the lice burdens within each group. This was due to the fact that only fish from the most extreme HR and LR families were to be included in the study, but since fish from all families were mixed in the sea cages, it was difficult to control which fish were sampled at a given time. Subdividing fish according

to lice burden (<10 lice or >10 lice) allowed for a more just comparison of responses. It also enabled us to separate responses due to family background away from lice burden. In paper II, lice density, and not lice counts *per se* was used to calculate the breeding value, since lice counts can increase with increasing body weight (99), in contrast to lice density (66). Although the use of genetic information to select breeding candidates is expected to outperform the use of pedigree-based models for identifying lice resistant and -susceptible individuals (67), the technology is still at an early stage for Atlantic salmon breeding. Furthermore, the goal of the study was to characterize differences in skin immune gene expression profiles between fish from high resistant (HR) and low resistant (LR) families, and not to study alternative methods for selective breeding.

4.2. Selection of tissues for various analyses

Various types of analyses were conducted in paper I-IV. On the phenotypic level, fish weights, condition factors and somatic indices were calculated in paper IV. Histological analysis of skin allowed for the studies on the local responses to lice infections (skin) (paper I-II), and level of fat deposition in fish fed functional feeds (paper IV). Immunohistochemistry of skin (paper I), and biochemical assays of plasma and NQC samples (paper IV) were also performed. On the transcription level, qPCR (paper I-IV) and microarrays (paper III and IV) were used to study changes in gene expression. A combination of analyses on different biological levels provided a better understanding of the observed changes.

In paper I, qPCR was used to profile the expression patterns of 32 candidate genes in skin from the occipital part of the head and in skin behind dorsal fin, the latter site was also used in paper II and III. In addition, histology and immunohistochemistry (IHC) techniques were used to identify AB-PAS$^+$, Mx$^+$, MHC class II$^+$ and CD8α^+ cells in skin from the occipital part of the head in paper I. This site was selected for performing histological techniques since it is free of scales. The presence of scales often results in skin detaching from the glass slide during the IHC procedure, which makes the identification and reproducible counting of positive cells challenging in our experience. A predefined selection of the skin sampling spot was chosen in paper I-III. This was done for several reasons. As RNA is prone to degradation after the fish is dead, it is important to harvest tissues quickly, and standardized protocols are necessary to ensure efficiency. Furthermore, skin composition differs on the fish body, at

the transcript and protein level (210, 440), which may affect the results. Thus, a decision was made to sample skin on top of head and skin behind dorsal fin for gene expression analyses, since we could expect host responses to lice infections at these sites. Copepodids are known to attach to the body in experimental studies (61, 97). Grimnes *et al*. reports epidermal reactions on the dorsal side of Atlantic salmon the day after copepodid exposure (6), gross lesions are commonly seen dorsally on the fish body with infection with chalimus stages (103), and on the top of the head and between the dorsal and adipose fins with infection with adult stages (102). The dorsal surface is a preferred site for lice attachment at the post-chalimus stages of farmed Atlantic salmon during natural infections (99), and in experimental studies (109). Furthermore, host gene expression responses in skin behind dorsal fin during experimental salmon lice infection with all lice stages, is reported (70, 124). Fins were not sampled, due to the common observation of fin damage in farmed Atlantic salmon in cages under different dietary regimes, which could affect the results (441).

In paper IV, analyses of liver, distal kidney and muscle by gene expression and histology (liver) were conducted. This study aimed at screening the possible positive and adverse effects of feeding high dose of Gls in Atlantic salmon. Liver was chosen due to its importance as an accessory organ within the teleost digestive system, involved in metabolism of fat, carbohydrates and protein and formation of bile. Equally important for paper IV was to study the detoxification properties of liver under Gls exposure, as fish liver is particularly susceptible to chemical damage (442). The liver is also an important storage location for energy reserves in fish, thus a set of measurements were done to address the energy status of the fish. Visualisation and scoring of fat accumulation in liver histology sections were performed. This was important to address as increased plant derivatives in salmon feed is known to affect the hepatic lipid content in mice models (443) and in Atlantic salmon (444). For this purpose, a steatosis scoring system from a similar study addressing the systemic effects of feeding functional feeds in Atlantic salmon was used (445). Furthermore, an estimation of the growth and energy status of the fish was also made by calculating the Fulton's condition factor (446, 447), which estimates the extent to which the total weight of a fish is high for its length (448). Organ indices are useful indicators of change in nutritional, health and energy status of fish. Therefore, weight measurements of liver and intestine in relation to the carcass; namely hepatosomatic and intestinal somatic indices was performed (448). Higher

energy reserves in liver results in higher liver weight, but enlarged livers can also be pathological, for instance due to contaminant exposure (449).

Distal kidney is the excretory part of the kidney, important for osmotic regulation of water and salts. Pathology in the excretory kidney is commonly seen during viral infections, pollution with heavy metals, with high levels of calcium and magnesium in the diet or exposure to toxic organic compounds. Muscle was important to analyse, as myopathies related to dietary defects are commonly reported (442). Muscle also responds to immune modulators in fish, *in vivo* (450) and *in vitro* (451). Muscle samples from the region of the Norwegian Quality Cut (NQC Norwegian standard procedure – NS 9401 1994) were also sampled for Near Infrared spectroscopy (NIR). Using spectroscopy techniques on NQC samples gives accurate information on the fatty acid composition in salmon (452). NIR is a spectrometric method for analyzing contents of a biological sample, using information of the chemical and physical composition gathered from the light absorbed by the sample. Lastly, this study used plasma biochemistry to analyse the content of ions and enzymes. Levels of plasma enzymes may be used for diagnosis and treatment of disease processes. We opted for analysis of bilirubin, cholesterol, alanine aminotransferase (ALT), aspartate aminotransferase (AST), creatine kinase (CK) and Na/K ratio. Bilirubin is a metabolic product of haemoglobin and released during breakdown of senescent erythrocytes. Conjugation of bilirubin happens in liver. With liver damage or increased haemolysis, bilirubin levels may rise in mammals (453). Cholesterol can be produced by the liver or gained from the feed. Variation in dietary lipid levels and lipid source may affect the plasma cholesterol levels in Atlantic salmon (454). Hepatic failure may cause changes in plasma cholesterol (455). ALT is found in kidney, heart, muscle and liver of humans. Increased levels are associated with diseases affecting hepatocytes in humans. AST is found in heart, to a lesser degree in liver, skeletal muscle and kidney of humans (456). Increased levels of ALT and AST are associated with exposure to toxic levels of metals in the water and liver toxicity in carp (457). CK is released upon cellular necrosis, in mammals this occurs from myocytes in skeletal or cardiac injury (458). CK levels increase during heart and skeletal muscle inflammation (HSMI) of Atlantic salmon, and suggested to be a useful biomarker for screening of this disease (459). Lastly, measurements of Na+/K+ ratio were performed. Stress causes ionic plasma disturbances in fish, and stress hormones have rapid actions on the branchial ATPase activities (460). An increase in plasma sodium

levels in salmon lice infected Atlantic salmon injected with cortisol has been found previously (282).

4.3. Microarray technology

Study III and IV used Nofima's Atlantic salmon 15k Salmon Immunity and Quality (SIQ) 6 oligonucleotide microarray technology produced by Agilent technologies. Analyses was interpreted by using Nofima's bioinformatics package STARS for data processing and mining (461). Microarray analyses measure the expression of large numbers of genes in parallel by containing probes designed to all unique transcripts available from public databases. The probes were selected with an emphasis on nucleotide sequences with a low redundancy but comprehensive coverage of the transcriptome. This was followed by identification of genes from searches in protein databases, or by functions, pathways or structural features (461). RNA isolated from two samples, a control sample or a technical reference control and a test sample, are reverse transcribed with incorporation of red and green cyanine dyes, combined and hybridized to the microarray probes. After performing several incubation and washing steps, subsequent scanning with laser measures the amount of dye in each spot on the microarray slide. The STARS database contains custom annotation of genes included on the microarray based on GO classes, KEGG pathways, mining of literature and public databases and experimental evidence (transcription profiles/meta-analysis) (461). The SIQ microarray technology platform has been used in a range of studies on Atlantic salmon host responses to bacterial (462), viral (463-466), and *L. salmonis* (70, 124, 125, 282) infections, which allows for easier interpretation of results. Furthermore, the STARS database is continuously updated after each release of UniGene. Validation of microarray results through qPCR is considered the gold standard (467), and was performed in both study III and IV.

4.4. qPCR

The candidate gene approach was selected in all papers by using real time quantitative polymerase chain reaction (qPCR) technology. This is an accurate and sensitive method, which allows many samples to be analyzed at once. The number of copies of mRNA transcripts in a cell or tissue is determined by the rate of expression and degradation. This qPCR technology is based on three steps: the reverse transcriptase (RT) dependent conversion of RNA into cDNA (cDNA synthesis), the amplification of cDNA using the PCR, and quantification of

amplification products. Fluorescence from a reporter molecule increases with each cycle of amplification. Fluorescence from each sample is measured once each cycle during the PCR, and at a certain cycle number, the fluorescence generated within a reaction crosses the fluorescence threshold, and the Ct (threshold cycle) value is the cycle number at which this happens. In other words, the Ct value is the intersection between an amplification curve and a threshold line fluorescent signal that is significantly above the background fluorescence. At the threshold cycle, a detectable amount of amplicon product has been generated during the early exponential phase of the reaction. The threshold cycle is inversely proportional to the original relative expression level of the gene of interest.

There are specific and non-specific fluorescent detection systems available (468). Specific systems includes various probes, which are complementary to the target sequence. SybrGreen is a non-specific reporter molecule, and widely used. Since SybrGreen is not sequence-specific, it can be used for any reaction, but the challenge is that it may bind to DNA residues and create primer-dimer artefacts, which lowers the specificity of the assay. This makes melting curve analysis necessary, to confirm specific amplification of product, and agarose gel electrophoresis analysis to confirm that the product is of correct size (468). For relative quantification, one analyses changes in gene expression in a given sample relative to another reference sample (such as an untreated control sample). qPCR relative quantification has been widely used to study immune responses in fish, and the commonly used method for calculation of the expression change is the –ddCt method (469). qPCR has the advantage of studying many genes of interest simultaneously. The selection of genes for a qPCR study is often based on the candidate gene approach, *i.e.* an "educated guess" based on hypotheses. The genes for studies in paper I-V were selected based to a large degree on microarray studies on Atlantic salmon responses to *L. salmonis* (70, 124, 125, 282), inter-species comparative studies on host responses to *L. salmonis* (55, 58, 59, 118), and salmonid responses to other parasites (150, 257), in addition to new gene candidates of interest. The MIQE (minimum information for publication of quantitative real-time PCR experiments) guidelines provides guidelines for the minimum amount of information which should be addressed when publishing qPCR data (470).

4.5. Histology and Immunohistochemistry

Histological techniques were used in paper I, II and IV. In paper I, the pH of the mucus cell content was evaluated with Alcian blue/Periodic Acid Schiff (AB-PAS) method, whereas enumeration and size measurement of mucus cells were performed in paper II with PAS staining. These staining procedures are commonly used for identifying carbohydrate-containing macromolecules, like glycoproteins called mucins that constitute the main part of the mucus cell, [as reviewed in (144)]. The epidermis of fish skin can vary in thickness related to lice infections (57), tank conditions (471) and temperature (472), therefore, size standardization of the area in which PAS^+ cells (paper II) or cells showing immunoreactivity (paper I) were measured or counted, were performed. In paper IV, PAS staining was also used to exclude that the vacuoles observed in hematoxylin and eosin stained liver sections were caused by glycogen storage instead of fat.

Immunohistochemistry (IHC) provides a way to identify cells positive for certain proteins at tissue level, and the positive cells can be counted. The major limitation with extensive use of IHC techniques in fish is the availability of antibodies. Molecular techniques such as qPCR can only provide information of the amount of mRNA transcript at a given time, but it is not certain that the transcripts will be translated into functional proteins. IHC on the other hand uses antibodies to detect positive cells for a certain epitope. Unspecific binding may result in high background and difficulty in identifying positive cells. A common cause of non-specific staining may include interactions of the primary and secondary antibodies with tissue proteins. Therefore, care should be taken to ensure reduction in non specific interactions without affecting the antibody-epitope binding. Normal serum is a commonly used blocking substance, since the serum contains antibodies, which bind to the tissue and prevent non-specific binding of the secondary antibody used in the assay. Serum from goat was used as a blocking agent in paper I. Furthermore, endogenous peroxidases, for instance present in inflamed tissue, may also result in unspecific staining. Inhibition of these was achieved by incubating tissue sections with phenylhydrazine. Antibodies may be directly or indirectly labelled to allow visualization of the antibody-antigen binding. Direct labelling involves using an antibody, which is complexed with a fluorescent or chromogenic label. Using a secondary antibody that binds to the primary antibody is called indirect labelling. This secondary antibody can be labelled with fluorescent dye, or with an enzyme or enzyme substrate, which

requires that a subsequent enzymatic colour reaction for developing the signal is used. The secondary antibody used in paper I was bound to the enzyme peroxidase that produces a positive red signal through an enzymatic chromogen reaction when 3-amino-9-ethyl carbazol (AEC) was added. Western blot is commonly used to validate the specificity of novel antibodies, which would mean observing a single band at the known molecular weight for the target. A range of other methods to validate the specificity, selectivity and reproducibility of antibodies in given in Bordeaux *et al.* (473). Validation of the antibodies used in paper I was performed elsewhere (211, 474-476).

There are a number of ways to perform IHC; the methods selected in paper I were based on in-house developed procedures, with optimization to fit the specific conditions. This was especially related to the antibody dilutions used, incubation time for primary and secondary antibody, and time used for development of signal. IHC is a method that is very sensitive to variations in the protocol (incubation temperature, time, thickness of tissue, quality of reagents), thus care was taken to ensure reproducibility of the results.

5. Results and Discussion

5.1. General discussion

An important limiting factor in salmonid aquaculture is fish diseases, due to poorer animal welfare, reduced growth, increased mortality, and higher cost of production. For the sustainable future of aquaculture, an integrated louse management approach should be emphasized. Gene expression technology made important contributions in the field in the last decade, and allowed for a deeper understanding of the relationship between the salmon louse and its hosts. The genomes of the salmon louse and Atlantic salmon are now both sequenced. Genome sequencing will enable development of tools for high-throughput screening of tissue transcriptomic responses. Functional genomics studies of both the host and the pathogen will likely aid in better understanding of their interactions, hopefully resulting in new solutions for lice management and control. Screening of the recently sequenced genome of *L. salmonis* revealed useful information related to iron metabolism. The genome apparently lacks some of the factors needed for making iron containing heme; thus the parasite probably largely depends on the host for iron and heme (Prof. Frank Nilsen, personal communication). Gene expression data presented in papers III and IV support this suggestion. The sequenced transcriptome and genome of Atlantic salmon allowed for microarray gene expression profiling of around 15 000 probes. It appears that iron withdrawal response involves several organs, and is one of Atlantic salmon endogenous responses to lice, aiming at depriving the lice of essential nutrients. Furthermore, the response was found affected by the tested functional feed.

The candidate gene approach in paper I and II was used to investigate a smaller number of most interesting genes by qPCR. The selection of genes was based on published papers, as the study field of host responses to salmon louse infections grew with the increasing use of functional genomics tools. The use of qPCR technology allowed for analyses of a larger number of fish, and selection of specific gene isoforms to study.

When studying host responses to salmon louse infections, it is important to take the infection level into account. In paper II, we found that the lice infection level affects the gene expression. In paper III, Gls exposure resulted in a significant reduction in the lice number;

however, some gene expression responses observed in paper III could be a result of differences in lice burden. As discussed in paper III, Gls can mediate a number of effects. Gls might be changing the physicochemical properties of skin/mucus, or be deposited in skin/mucus and act as a repellent. Gls were also shown to stimulate skin immune responses. All of this may interfere with the host recognition and the attachment process of the parasite, thus resulting in lice reduction at the very beginning of infection. Smaller number of attached lice would mean that smaller amount of immunomodulatory substances are released onto the host. It could be expected that the less disturbed host would consequently be better at avoiding immunosuppression and answer by mounting a more effective immune response. Future studies should make an attempt to count lice at one or two earlier time points during the infection period in order to assess when most of the feed mediated protection occurs.

Challenge trials with well-controlled experimental conditions are different from natural infections occurring under aquaculture conditions. In the field, a greater number of parameters exist that cannot be controlled and they may also change more dynamically than under lab settings. The best validation of the dietary effects against a disease is through both controlled lab challenge trials and field studies (477). The feeds used in paper III and paper IV showed promising effects in the lab, and make good candidates for future field tests. The results in paper II were obtained from a challenge trial performed at an experimental facility in net cages in the sea, thus milieu parameters were less controlled. However, the infection was not natural; the infection procedure was conducted by adding *L. salmonis* copepodids to the water in net cages closed by tarpaulins. In natural settings, fish can be infected with several lice stages at the same time.

The number of attached lice is a continuous phenotypic trait, and high variation in lice numbers is commonly seen (65, 66, 99, 362, 363, 478), which means that a large number of fish needs to be phenotyped to be able to calculate the genetic component in the trait and rank families into resistant and susceptible. Decreasing the number of experimental fish would be in line with the principle of the three Rs (Replacement, Reduction and Refinement), for the use of animals in experimental testing (479). The reduction principle involves that researchers select methods that can obtain comparable levels of information from fewer animals. In the future, selective breeding models based on genomic information will make the selection of breeding candidates a lot less extensive (67). In paper II, selection of individuals

used in the study was based on lice burden at family level. Given the moderate magnitude of protection conferred by breeding and big environmental effects at play, the study would benefit from a larger number of fish per family sampled for gene expression analysis.

5.2. Host responses during the copepodid and chalimus stages

In paper I-III, we observed gene expression and IHC responses in skin and internal organs at specific time points. Responses that occur within the first 5-10 days of infection determine the resistant phenotype in Pacific pink and coho salmon (57, 59, 60). Better understanding of early host responses to lice in Atlantic salmon is also needed. Reduced number of chalimii and adult stages of lice measured in our trials (paper II and III) may be the result of protective host responses mounted to previous stages. In paper I, gene expression responses around the time of copepodid and moulting to chalimii were addressed. Suppressed transcript level at 4 dpi in skin was the norm for the majority of genes. At 8 dpi, the suppression was less pronounced, but present for the majority of genes, as few genes managed to surpass the not infected control level. It is tempting to speculate that this early suppression is mediated by SEPs excreted by lice. In paper III, increased transcript levels of interferon related genes, chemokines and cytokines, acute phase proteins, antimicrobial proteins, lectins and complement factors coincided with reduced number of preadult and adult lice 5 weeks into the infection. Several of these were also correlated with reduced lice number in paper II.

In paper I, we found that Atlantic salmon epidermis is rich in MHC class II$^+$, CD8α$^+$ and Mx$^+$ cells. We also found a small, but significant increase in the number of CD8α$^+$ cells at 8 dpi compared to not-infected fish. The CD8α$^+$ cells were compartmentalized together with the Mx$^+$ cells along *stratum basale* in skin. CD3ε$^+$ cells are also found located in the same location (Helle Holm, unpublished results) (Fig. 9), and recently, CD3ε$^+$ cells were found along the basal cells in rainbow trout skin (210), suggesting that this specific location is important for mounting T cell mediated responses in salmonid skin. In addition, paper I found MHC class II$^+$ cells scattered evenly throughout the epidermis, including keratinocytes. Thus, the epidermis of Atlantic salmon contains important components, which can launch an innate and adaptive immune response, including dendritic cells (MHC class II$^+$ cells and CD8α$^+$ cells), which can activate T cells (211, 480), T cells (CD8α$^+$ cells (210, 474) and Mx$^+$ cells. Mx$^+$ cells were abundant, and positive cells included keratinocytes, mucus cells and possibly lymphocytes, as strongly positive Mx$^+$ cells were found in the same location as the CD8α$^+$ and CD3ε$^+$

cells (Fig. 9). Further studies should attempt co-staining using the antibodies CD8α⁺, CD3ε⁺, and Mx, to be able to address whether the immunoreactivity originates from the same population of cells.

Activation of interferon responses is usually associated with viral infections. One unexpected finding in paper I was observing positive Mx reaction throughout the epidermis, in non-infected fish. Fish are constantly exposed to microbes in their aquatic surrounding, thus likely their skin immune barrier needs to be adapted to a higher level of microbial exposure.

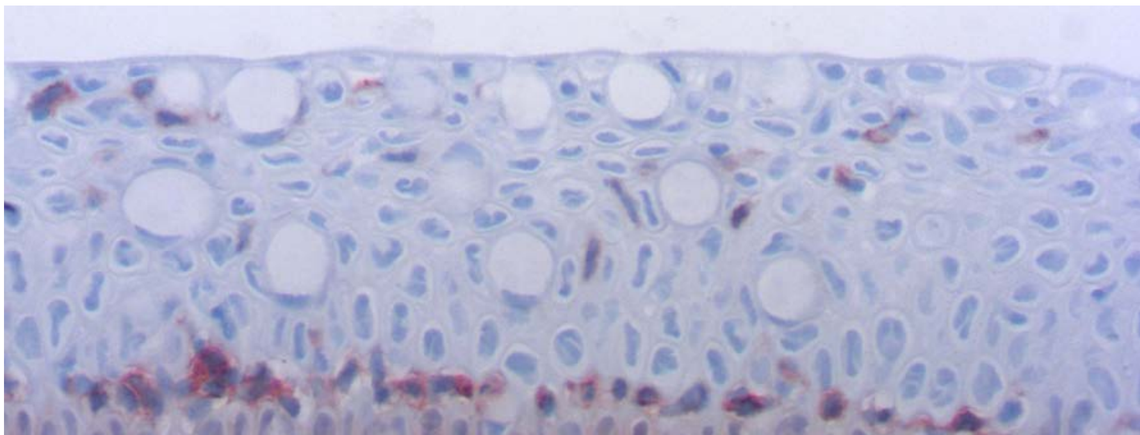

Fig. 9 CD3ε⁺ cells in bright red along *stratum basale* in Atlantic salmon skin 8 dpi after *L. salmonis* infection. Helle Holm, unpublished.

5.3. Protection mediated by selective breeding

The selection of genes in paper II was based on published studies that investigated skin responses to lice. In paper II we investigated responses in skin of individuals selected for increased and decreased resistance to lice. In addition, we addressed the relationship between number of attached lice and gene expression. Lice number shows a large phenotypic variance, it is a continuous and quantitative trait, influenced by numerous genes and environmental factors. The fish groups need to be compared in similar conditions, and this was performed in paper II, as the impact of milieu can mask the effects of the underlying genotype. Cohabitation of fish was performed in sea net cages, where copepodids were added directly into the water enclosed by a tarpaulin. The non-standardized conditions could be the reason for the lower estimated heritabilities compared to what was found in previous studies (65, 66). High variation in lice burden is commonly seen in wild and farmed fish (46, 67, 363). Glover *et al*. found no relationship between the parasite burden in two consecutive lice challenges, and suggested that major environmental and random factors contribute

greatly to the number of attached lice on a given fish (347, 363). This means that in a selective breeding program, a large number of fish needs to be phenotyped in order to determine the genetic effect on the trait and to rank families into HR and LR. Interestingly, a QTL for lice susceptibility was recently suggested in Atlantic salmon stocks from the same company (AquaGen) that the fish in paper II originated from (372). Using genomic information in selective breeding programs may be more accurate for selecting the best breeding candidates, and reduce the number of breeding candidates that have to be tested, in line with the 3Rs tenet, Replacement, Reduction, Refinement (479). In a paper by Ødegård *et al.* that used data produced from the same challenge as in paper II, genomic selection models turned out to be more reliable for lice density than the pedigree based model (67).

In order to accomplish the required accuracy, the candidate gene approach by qPCR profiling was selected in paper II, as the differences were expected to be small to moderate. The use of qPCR technology also allowed for the analyses of a larger number of fish, and more specific selection of gene isoforms to study. On the other hand, the candidate approach does not encompass all potential markers of resistance to lice. For instance, differences in the regulation of iron related transcripts in head kidneys of Atlantic salmon were not investigated, but in families with varying resistance/susceptibility towards piscirickettsiosis these differences were found to be important (481). These mechanisms could also play a part during *L. salmonis* infections, as will be discussed in 5.4. Although Atlantic salmon is considered susceptible to *L. salmonis* infections, a number of selective breeding trials found a low to moderate level of heritability (65, 66, 344). This suggests that there is enough additive variation within the species that can be used to increase resistance through selective breeding (66). Differences between families for a certain phenotypic trait may be large, for instance body weight were found to differ by 2.6 kg among 13 full-sib families of Atlantic salmon (482). Similarly, a 70 % variation in infection level between families was found for salmon lice (363).

The ranking of families into HR and LR was based on two full-sibling tests on 150 families, including over 5000 individual fish. Full-sibs share half of their gene alleles, and family selection is regarded as good for traits with low heritability, since the phenotypic mean of the family is a good measure of its genotypic mean, and environmental effects tend to erase each other away in the mean value of the family (348). The ranking of families in paper II

was based on breeding values of lice densities, which included information of lice density from the fish itself and its relatives. In the fish selected for analyses in paper II, the reduction ($P < 0.05$) in mean lice numbers in HR compared to LR families (5 families from each) was 36 % and lice density was reduced by 31 %. However, individuals within LR and HR groups showed large variation in lice counts, suggesting a strong influence of factors not directly related to the fish genetic background.

By using a candidate gene approach and applying multivariate statistics in paper II in addition to morphometric analyses of the skin, we found that there is gene expression variation between the individuals belonging to high resistant HR families and low resistant LR families. Subdivision of HR and LR families into groups that have more or less than 10 lice, enabled us to understand better to what extent the number of attached lice may affect responses caused by family genetics. We found that HR fish were better at resisting lice-induced suppression. This included a group of genes that may be involved in type 2 responses, and interferon and other type 1 responses. Inverse correlation between the number of attached lice and gene expression was found, in line with previous studies suggesting that lice potently immunomodulate their hosts. On the other side, we found that HR fish, especially fish with less than 10 lice, were better at maintaining a higher expression level of Th1/type 1, and to a certain extent Th2/type2 markers of immunity. Activation of type 1 immunity was also linked to protection against lice in paper III, in lice resistant coho salmon (56) and in Atlantic salmon fed 17β-estradiol, which gave significant reduction in lice counts (70). LR fish had larger mucus cells and thicker epidermis behind the dorsal fin but still harbored more lice. Immunity appeared more important for resisting lice infections than physical barrier functions.

5.4. Protection mediated by glucosinolates-based functional feeds

The use of feed additives is a promising approach in aquaculture for modulation of endogenous protective responses, including the effects on the immune system. Different plant derivatives increase innate immune activities and increase resistance to *Aeromonas hydrophila* in tilapia (483) and goldfish (484) and lymphocystis disease virus in flounder (485). In paper III, the plant feed ingredient containing bioactive phytochemicals (Gls) affected interferon-mediated responses. Induction of immune genes in non-infected skin in fish exposed to Gls could be a kind of preconditioning of the skin immunity that later in the challenge test leads to reduced number of attached lice. The group of activated genes included 66 interferon related genes that could be playing a role in the first line of defence, soon upon the encounter of the parasite. Interferon-related genes were more highly expressed in infected fish exposed to Gls, which had fewer lice than control. IFNs also regulate the subsequent T cell responses. Mammalian studies show that IFNs contribute to T cell expansion and Th1 commitment (243, 244, 486). Secreted type 1 IFNs result in increased transcription of a number of genes, named Interferon–Stimulated Genes (ISGs) including *mx* (487). Some of these were anti-correlated to the lice number in paper II, and upregulated in Gls exposed groups in paper III.

One way functional feeds could work is through disruption of the early host location and probing period of the copepodid. This could be achieved by masking or changing the smell of the host, as suggested in paper III. Semiochemical cues emitted from the host are vital for proper host location by the parasite (94). Salmon conditioned water contains low molecular weight compounds, which can be isolated by vacuum distillation and GC-MS analysis (149). Manipulation of semiochemical host location cues have previously been suggested as a possible means to reduce salmon lice infection pressure (147, 149, 488). Host-preference experiments using Y-tubes have shown *L. salmonis* larvae display a kinetic behavioural response to seawater conditioned with the Atlantic salmon skin, mucus and flesh substances, compared to that of turbot (*Scophthalmus maximus*) (93). After initial contact with Atlantic salmon, the louse is probing the skin of the host, and this settlement is reversible (87). It may be that the louse continues the decision process before commencing its development (Simon Wadsworth, personal communication). For instance, internal frontal filament material was first observed at 2 dpi in copepodids infecting Atlantic salmon, and external frontal filament was first observed at the chalimus stage (77). Also after attachment, lice behaviour

is different among salmonids. Adult lice exhibit an exhaggerated transcriptional feeding response when attached to Atlantic salmon, whereas responses in Pacific salmonids showed characteristics of a starvation response (54). Similarly, the release of proteases from the salmon louse is largest in susceptible hosts (121), and lice grow faster on Atlantic salmon than on the resistant coho salmon (57). Thus, disturbing lice chemoreception of the Atlantic salmon host, by modulating the skin and mucus composition through the use of functional feeds would be very useful. Paper III suggested specific genes possibly related to skin composition parameters that should be further studied.

Dietary activation of the host nutrient deprivation responses, which withhold important nutrients from the parasite may be another mechanism of protection that can contribute to lice reduction. Lice appear to lack part of necessary components for the syntheses of heme, and possibly also cholesterol (Prof. Frank Nilsen, personal communication). Regulation of iron metabolism, on the transcriptional level, is reported in a range of studies (56, 59, 70). The resistant pink salmon initiate a regulatory iron sequestration response that likely results in the iron/heme deprivation of the parasite (59). We observed in paper III induction of iron regulators in skin of the Gls fed group of fish. In paper IV, hepatic induction of *hepcidin-1*, which regulates the release of iron from tissue macrophages and hepatocytes was found. In distal kidney, the iron sequestrator *ferritin* was induced. Altogether, it seems that activation of the iron sequestration responses in multiple tissues may play a role in protection by depriving lice of iron and heme, and these responses can be stimulated by diet.

Lice infections are associated with open wounds, especially with preadult and adult lice stages (6). Resistant coho salmon are able to proliferate the epidermis below the site of attachment and feeding, forming what appears to be a physical protective barrier (60). On the host transcript level, differential regulation of genes involved in the wound healing cascade was found in a range of studies in Atlantic salmon infected with chalimus, preadult and adult lice stages (70, 124, 125, 282), and were also found in paper III. Gls fed groups of fish showed induction of *mmp13* and keratins and downregulation of collagens and genes with roles in tissue differentiation, formation of extracellular matrix (ECM) and wound healing at 5 weeks post lice infection. Paper III also found similar results to those reported by other authors (124, 125, 282), namely that the regulation of myofibers and glycolytic enzymes occurs in skin infected with salmon lice. Paper III, as already discussed, suggests that type 1

immunity has a stronger contribution to protection. However, improving wound healing properties at the same time could be an interesting approach in future research on functional feeds.

Adverse affects of functional feeds need to be considered before their wider practical use. Too high Gls doses may cause lower growth in Atlantic salmon as seen in Trials 1 and 2 in paper IV. Lower inclusion levels appear as safe options as they showed reverse trend on growth in Trial 3. Comprehensive analysis in paper IV revealed a number of factors that likely reflect reduced metabolic tissue activity and growth. Microarray gene expression analyses suggested low cellular proliferation in liver and muscle of fish fed high dose of Gls. Gls showed a lowering effect on liver steatosis in all trials. Hepatosomatic indices and liver steatosis decreased even at the lower Gls levels in Trial 3. Induction of responses in the distal kidney against renal injury was suggested by the transcriptome data in the group receiving highest dose of Gls. However, plasma levels of several indicators of cell leakage in liver, kidney, muscle and other tissues (ALT, AST and CK) did not capture any significant differences between the control group and the group overexposed to Gls. Finally, the microarray data showed that the antioxidant and detoxification status in studied tissues are likely improved by Gls, and may contribute to the overall health status of the fish.

5.5. Integrated pest management control

Chemical treatments should be sparingly used as a control resource for sea lice. Thus, there is a great interest for developing alternatives to chemical treatments for management and control of sea lice. Application of integrated pest management (IPM) strategies involving several tools to manage salmon lice problems should shape the future anti-lice strategies. At present, Norwegian salmon farming relies heavily on the use of chemicals to control lice levels on farms, but the use of alternative treatments is on the rise. In their report published in the summer of 2016, the Norwegian Food Safety Authority reports of 198 cases of delousing events in 2016, where reduced fish welfare, physical trauma and mortalities have occurred, especially following immediate measures such as mechanical treatments (13) (Fig. 10). That is not acceptable.

A proper foundation for the IPM strategy (Fig. 10) constitutes preventive measures, including vaccination and other biological control measures, such as selective breeding and functional feeds (40). However, none of these approaches should be a stand-alone measure, since none can provide a full eradication of lice. Rotation of several active ingredients in the feed might be important. Overuse of a few chemotherapeutics has been shown to cause development of resistance (20). Proper dosage of bioactive ingredients in functional feeds appear as important, as well as the timing of administration of these feeds during the year. In paper III, 25 % reduction in lice counts were observed in fish fed the low dose of GIs. AquaGen selective breeding program achieved a fairly moderate effect on the lice resistance trait. It must be assessed whether selection for increased resistance affects other important traits in Atlantic salmon. The estimates of genetic correlation between survival after viral and bacterial disease may be negative or positive (489), and should be taken into account. It appears that functional feeds and selective breeding can be used together. In both paper II and paper III, reduced number of lice coincided with a higher expression of interferon related genes and markers of type 1 and type 2 immunity. Feeding functional feeds to HR fish may provide an additional boost to skin immune defences, however timely implementation needs to be made in order to not risk affecting the responses to other diseases. Furthermore, it needs to be checked if the two approaches combined would produce additive results.

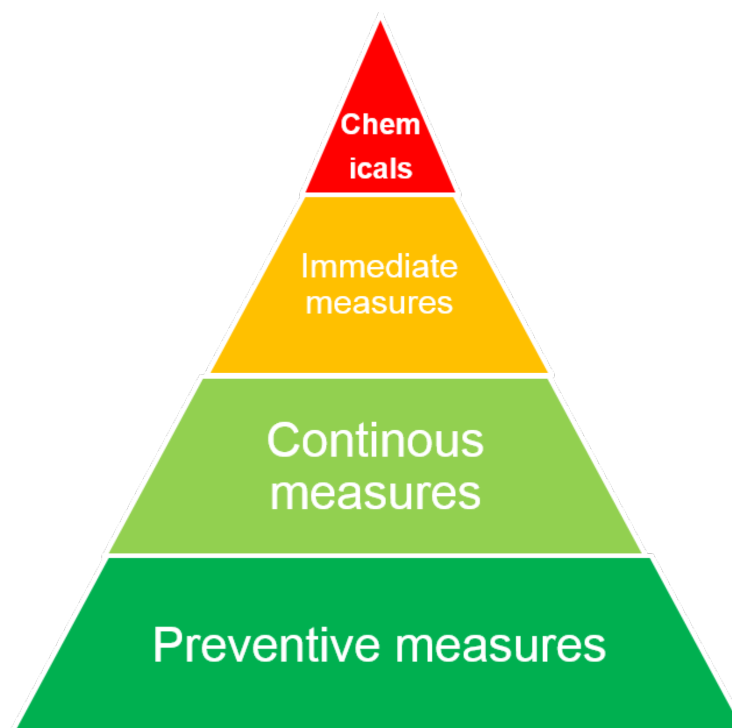

Fig. 10 Integrated pest management approach for sea lice management and control. Reused with permission from Randi Grøntvedt.

6. Main conclusions

This thesis contributed to our understanding of Atlantic salmon responses to *L. salmonis*. It also outlined how beneficial endogenous responses can be modulated by selective breeding for increased resistance to lice and by exposure to anti-lice functional feeds. In paper I, previously not described skin tissue localisations of immune factors were assessed, and immune responses following copepodid attachment were reported. Skin immune transcript levels coinciding with protection mediated by selective breeding were shown in paper II, and several skin histological parameters were shown as well. The role of multiple organs and mechanisms behind protection conferred by Gls enriched functional feed were discussed in papers III and IV.

- Epidermis of Atlantic salmon contains factors with putative roles in the adaptive arm of immunity. MHC class II^{+} and Mx^{+} cells are widely dispersed throughout epidermis. The role of MHC class II^{+} keratinocytes as initiators of adaptive immune responses should be further studied. Strongly immunoreactive Mx^{+} cells were located together with CD8α^{+} cells along the *stratum basale*, possibly forming an important immune barrier.
- Skin sites show differences in the constitutive expression levels of immune related transcripts. However, gene expression responses during development of copepodids (4 dpi) to chalimi (8 dpi) were mostly similar at two sites; immune gene expression in skin infected with copepodids was largely suppressed, with a temporal increase towards 8 dpi in expression of most studied innate and adaptive immune markers.
- Significant reduction of lice number is possible through the use of selective breeding in Atlantic salmon. Resistant animals appeared better at avoiding lice-mediated suppression, and had higher expression profile of type 1 immune related genes, and to a certain extent, higher expression of genes encoding type 2 immune factors.
- Gls fed fish showed significantly lower lice counts. Growth and organ function could be compromised by the high dietary dose of Gls. Pathways and genes in skin, liver, muscle and distal kidney potentially associated with protection against lice and other benefical health consequences conferred by the Gls-containing functional feed were proposed.

- Feeding Gls coincided with an increased expression of IFN-related genes, antimicrobial and acute phase proteins, in not-infected and infected fish.
- Iron related genes were affected by the Gls-enriched feed in a way that can be seen as beneficial upon encountering the parasite. The proposed iron/heme sequestration response could be an additional resistance mechanisms at play, apart from immunity.
- Similar immune mechanisms seem to play a part in selectively bred fish and fish fed Gls. Time course studies of the host responses to all lice stages in fish fed FF or in selective breeding trials will likely give more knowledge on which responses are most protective at each time point.

7. Future perspectives

Developing functional feeds that can reduce the number of attached lice and/or slow/prevent parasitic development is a promising tool in the integrated pest management of *L. salmonis*. Feeds enriched in phytochemicals may be a safe and cost-efficient option. However, a major limitation is the identification of bioactive candidates. Screening phytochemicals requires methods that can predict potential biological effects (and side-effects) on the host and the parasite. Promising feed ingredients/ bioactive molecules may have several modes of action. They can interfere with the chemotactic activities of the infective stages of louse, for instance by masking the smell of the fish by being accumulated in mucus and skin, or by changing their composition. Another mode of action that should be further explored is through affecting the nutrient deprivation mechanisms against lice (such as the iron sequestration response), making the host less nutritious and therefore less attractive for attachment and development. The data showed in this thesis and literature published so far strongly suggests that modulation of host immunity (steering it away from immunosuppression towards type 1 immunity) should remain an important line of research, both within the selective breeding and functional feed approaches. Mapping tissue localisation of some of the key immune players would help the interpretation of gene expression data. Quantification of changes caused by protective interventions (breeding and feeds) on a protein level would further refine our understanding of protection mechanisms. Addressing the role of specific immune pathways is a logical next step, and IFN-mediated pathways appear as a good candidate. In line with the 3Rs principles, excessive use of animals for experiments must be avoided. Technology exploiting genomic information for selecting good breeding candidates may provide an alternative to experimental challenges involving a large number of fish.

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Errata

Proofreading of the document and reference list, which includes corrections of misspellings and incorrect and imprecise grammar, has been performed. The manuscript has also been formatted to fit requirements for printing (letter size, page margins). In addition, the following changes have been made:

Sammendrag

Line 9, page 5: “stort” used instead of “viktig.”

1. Introduction

Line 8, page 12: added “affect” to the sentence.
Line 20, page 12: sentence is changed to “Good and dynamic husbandry and management techniques are important for controlling salmon lice levels on farms” from “Management of salmon lice by dynamic modification of the husbandry is important.”
Line 11 and 12, page 13: the word “thermolicer” is replaced by “hot water”, and “laser” is replaced by “optical delousing”, as “hot water” and “optical delousing” are the synonymous terms used later in the thesis.
Line 27, page 14: “salmon lice” is added to the sentence.
Table 1: row 16 and 19 contained similar information, and are now merged together in row 16. Row 16 contained the wrong reference, it is now updated (Dawson *et al.* 1999).
Line 20 and 21, page 31: sentence is changed to “In teleosts, metachrome staining of mast cells is observed using the same protocol recommended for identification of mammalian mast cells” from “In teleosts, mast cells stain metachromatically using the same protocol recommended for identification of mammalian mast cells.”
Line 6-8, page 32: sentence is changed to “Histopathological assessment of attachment and feeding sites in fin and gills of Atlantic salmon revealed minor host responses at the copepodid stage, similar observations through the juvenile stages in fin, gills and skin are also reported” from “Histopathology of attachment and feeding sites in in fin and gills of Atlantic salmon show minor host responses at the copepodid stage and also through the juvenile stages in fin, gills and skin.”

Line 21, page 32: the words “A microarray study of *L. salmonis* infected Atlantic salmon tissues” removed from the sentence, and replaced by “This study also”, as the information in this part of the paragraph concerns the same citation as the sentences preceding this one.
Line 11, page 36: reference was lacking and is now included at the end of the sentence.
Line 23, page 37: “In another study” added to the sentence.
Line 19, page 38: wrong citation used. Correct is Krasnov *et al.* 2015.
Line 21-22, page 39: sentence is changed to “Vaccines could be a cost-effective approach for controlling sea lice. In contrast to chemotherapeutants, vaccines can offer a prolonged duration of action and no withdrawal time” from “Vaccines could be a cost-effective approach for controlling sea lice, including sustained actions and no withdrawal period as for the chemotherapeutants commonly used.”
Line 27-28, page 39. “Several research groups have attempted to make a vaccine based on salmon louse gut antigens” from “Developing a salmon louse vaccine from gut antigens have been attempted by several research groups.”
Line 31 and 32, page 39: wrong citation used. Replaced with correct citation (Raynard *et al.* 2002).
Line 31-33, page 39: wrong citation used, replaced with correct citation (Roper *et al.* 1995).
Line 2 and 3, page 40: added “rat antibodies made from.”
Line 10, page 40: “*C. rogercresseyi*” added to the sentence.
Line 9-12, page 45: sentence is changed to: “In another study of rainbow trout, reduced growth after 3 weeks and thyroid disturbances after 9 weeks of feeding was found in fish fed Gls, shown by lower plasma levels of T3 and T4 and a hyperactivity of the thyroid follicles” from “In rainbow trout, reduced
growth after 3 weeks and thyroid disturbances after 9 weeks of feeding was found in fish fed Gls, shown by lower plasma levels of triiodothyronine and thyroxine and a hyperactivity of the thyroid follicles.”

3. Summary of Papers

Line 12, page 50: “named glucosinolates” added to the sentence.

4. Methodology

Line 6, page 53: “On the morphological level” removed from the sentence.

Line 29-30, page 53: “of farmed Atlantic salmon” added to the sentence. Wrong citation used at the end of the sentence, it is now updated (Dawson *et al.* 1999).
Line 30-32, page 53: sentence is changed to “Furthermore, host gene expression responses in skin behind dorsal fin during experimental salmon lice infection with all lice stages, is reported ” from “Furthermore, host gene expression responses to all lice stages during experimental salmon lice infections in skin behind dorsal fin is reported.”

5. Results and Discussion

Line 6, page 62: sentence changed to “In addition, we addressed the relationship between number of attached lice and gene expression” from “In addition, we addressed number of attached lice on gene expression.”
Line 1-3, page 63: sentence is changed to: “In order to accomplish the required accuracy, the candidate gene approach by qPCR profiling was selected in paper II, as the differences were expected to be small to moderate” from “Because the differences were expected to be small to moderate, in order to accomplish the required accuracy, the candidate gene approach by qPCR profiling was selected for the study described in paper II.”

Article IV

Correct labelling of groups in Figure 4 and 5 are now added below the figures.

Scientific papers

I

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Contrasting expression of immune genes in scaled and scaleless skin of Atlantic salmon infected with young stages of *Lepeophtheirus salmonis*

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ABSTRACT

Atlantic salmon skin tissues with and without scales were taken from two preferred sites of salmon louse (*Lepeophtheirus salmonis*) attachment, behind the dorsal fin (scaled) and from the top of the head (scaleless), respectively. Tissues were profiled by qPCR of 32 genes to study responses to copepodids, 4 days post infection (dpi), and during the moult of copepodids to the chalimus stage, at 8 dpi. Basal/constitutive differences were found for many immune-related genes between the two skin sites; e.g., *mannose binding protein C* was over 100 fold higher expressed in the scaled skin from the back in comparison to the skin without scales from the head. With lice-infection, at 4 dpi most genes in both tissues showed lower values than in the non-infected control. By 8 dpi, the majority of responses increased towards the control levels, including cytokines of Th1, Th17 and Th2 pathways. Immunohistochemistry of three immune factors revealed an even distribution of MHC class II positive cells throughout epidermis, including the top layer of keratinocytes, marked compartmentalization of Mx^{+} and $CD8\alpha^{+}$ cells close to *stratum basale*, and an increase in numbers of $CD8\alpha^{+}$ cells in response to infection. In conclusion, suppression of immune genes during the copepodid stage likely sets off a beneficial situation for the parasite. At the moult to chalimus stage 8 dpi, only few genes surpassed the non-infected control levels, including CD8α. The gene expression pattern was reflected in the increased number of CD8α expressing cells, thus revealing a relatively minor activation of skin T-cell defenses in Atlantic salmon in response to *L. salmonis* infection.

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1. Introduction

The salmon louse *Lepeophtheirus salmonis* (Krøyer) is the most important parasitic threat to sustainable Atlantic salmon (*Salmo salar*) farming in the Northern hemisphere. At present, control of caligid copepods is largely dependent on chemotherapeutants; expenses related to management and control of the parasite were recently calculated to 4 billion NOK (440 million Euro) annually in Norway (Iversen et al., 2015). In addition, there are concerns that a high number of infective stages of lice emitted from farms negatively influences wild salmonids (Costello, 2009). An increasing number of reports of reduced efficacy of the anti-parasitic chemical treatments is worrisome (Aaen et al., 2015). In Norway, there are strict control regulations on the maximum allowed parasite burden per fish (NFD, 2012). The parasite feeds on mucus, epithelial tissues and blood of host fish, which left uncontrolled leads to chronic stress, reduced growth rate, anemia, increased risk of secondary infections, and in worst cases osmotic failure and death in smaller fish due to compromised functions of epidermis (Dawson, 1998; Grimnes and Jakobsen, 1996; Igboeli et al., 2014; Johnson et al., 2004; Pike and Wadsworth, 1999). There is a growing body of evidence indicating that sea lice immunomodulate their hosts to their benefit by secreting/excreting a mix of proteases and other bioactive molecules (Fast et al., 2004; Firth et al., 2000; McCarthy et al.,

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2012). The amount of the secretory/excretory products is dependent on the host species, being highest on susceptible hosts (Fast et al., 2003). Atlantic salmon is considered the most susceptible species characterized by prolonged retention of parasites, in contrast to Pacific salmonids pink and coho salmon that show reduced lice burden already 5–14 days after the initial attachment (Fast et al., 2002; Johnson and Albright, 1992; Sutherland et al., 2014). Environmental factors strongly influence the outcome of salmon lice infections, with high variation in infection outcomes commonly observed in experimental lab trials and under farmed settings (Gjerde et al., 2011; Kolstad et al., 2005).

In the resistant species, quick rejection of lice was linked to activation of iron sequestration mechanisms (Sutherland et al., 2014) and an early development of local inflammation near the attachment sites, with high levels of infiltrating immune cells and proliferation of cells in the epidermal layer (Johnson and Albright, 1992). Recent studies suggest that T helper (Th) cells play a role in determining the type of inflammatory response (Braden et al., 2015; Holm et al., 2015; Skugor et al., 2008). Th-mediated responses are essential part of mucosal immunity that controls parasitic infections in mammals (Allen and Sutherland, 2014), while in salmon, there are several studies showing T-cell related transcripts being differentially regulated in salmon during parasitic infections (Benedicenti et al., 2015; Chettri et al., 2014; Kania et al., 2010) including sea lice infections (Braden et al., 2015; Holm et al., 2015; Krasnov et al., 2015; Skugor et al., 2008; Sutherland et al., 2014; Tadiso et al., 2011). A recent transcriptomic study suggested protective roles for an early pro-inflammatory Th1 response followed by the activation of Th2 pathway in skin of resistant coho salmon (Braden et al., 2015). The subdued inflammatory responses to lice typically seen in Atlantic salmon can be experimentally modulated by sexual hormones (Krasnov et al., 2012), but also through selective breeding (Holm et al., 2015) and functional feeds (Jodaa Holm et al., 2016), resulting in reduced infection levels. The work on Atlantic salmon selected for increased resistance to lice revealed that induction of antimicrobial immune responses in skin, broadly defined as Type 1, including interferon-mediated immunity, are most strongly correlated to reduced parasitic load, with Th2 or Type 2 responses likely also contributing in protection albeit to a lesser extent (Holm et al., 2015). Protection achieved by functional feeds (Jodaa Holm et al., 2016) and sexual hormones (Krasnov et al., 2015) also indicated that local activation of Type 1 immunity, including numerous genes from the Th1 and T17 pathways, promotes lice rejection in an otherwise susceptible host. The referred studies investigated responses to the chalimus and preadult lice stages, while the aim of this study was to learn more about the type of immunity activated in response to younger louse stages. A number of previous studies described the *L. salmonis* preferred sites for attachment and feeding activities (Dawson, 1998; Grimnes and Jakobsen, 1996; Jaworski and Holm, 1992; Johnson, 1993; Jonssdottir et al., 1992; Todd et al., 2000; Tucker et al., 2000; Tully et al., 1993) however, immune gene dynamics at different skin sites in Atlantic salmon have not yet been described. Differences in the distribution of innate and adaptive immune markers at different locations in skin of flounder, cod and rainbow trout have been found (Caipang et al., 2011; Leal et al., 2016; Nakano et al., 1993). Here, we report mRNA levels of 32 immune-related genes at two preferred sites for lice settlement in skin. The two locations, scaled skin behind the dorsal fin (DF) and the scaleless skin from the occipital part of the head (HS) were selected because of their importance as preferred sites of attachment. It also enabled us to address the compartmentalization of immune response, in particular, those related to innate immunity, Th cell pathways and cytotoxic T cells. In addition, to aid the interpretation of gene expression in the scaleless skin, we investigated the distribution of MHC class II^{+}, Mx^{+} and $CD8\alpha^{+}$ cells by immunohistochemistry in this area.

2. Materials and methods

The experimental facilities used in this study at Ewos Innovation, Dirdal, Norway, number 131 was approved by the Norwegian Animal Research Authority 02.02.2012 until 25.01.16. The experiments/procedures have been conducted in accordance with the laws and regulations controlling experiments/procedures in live animals in Norway, e.g. the Animal Welfare Act of 20th December 1974, No 73, chapter VI sections 20–22 and the Regulation on Animal Experimentation of 15th January 1996.

2.1. Production and establishment of copepodid cultures in the laboratory

Salmon lice (*L. salmonis*) used in this trial were a mixture of two strains; one wild-type strain collected from Oltesvik (Norway) in March 2012 and one laboratory strain (Ls Gulen) established in 2006 at University of Bergen. This lice population was propagated and maintained on Atlantic salmon hosts kept in the *L. salmonis* cultivation system in the louse lab at the Ewos Innovation's Test Facility in Dirdal, Norway. Lice and host fish were held in 850 L circular flow through tanks and egg strings from egg-bearing females were collected from anaesthetized salmon. The anesthetic used was Finquel (100 mg/l, Scan Aqua, Årnes, Norway). The egg strings were allowed to hatch and reach the infective copepodid stage at 9 °C for 14 days. The copepodid density was estimated in a zooplankton-counting chamber in four parallel water samples of 50 ml each, to improve the accuracy of estimation.

2.2. In vivo challenge

The trials were performed at Ewos Innovation's Test Facility in Dirdal, Norway. Fish (n = 300) (hatched autumn 2014) with an estimated weight of 484 g were randomly separated in 10 500 L tanks with an average water temperature of 8.5 °C and salinity of 28.3‰. At the day of challenge, fish were infected with 70 copepodids per fish in six parallel tanks. Water flow was turned off and water level lowered to 15 cm height before the copepodids were evenly distributed to the fish tanks. Oxygen was added using a fine ceramic diffusor, with individual air valves controlling the oxygen flow to each tank. After 1 h of exposure, water flow was resumed. Four tanks of fish were left non-infected (non-infected control (NI-C)) and skin tissues were sampled from these fish at 6 and 7 dpi for analyses. After 4 days post infection (dpi) (copepodid stage) and 8 dpi (copepodid to chalimus moult), 5 fish from each tank were anaesthetized with an overdose of Finquel (100 mg/l, Scan Aqua, Årnes, Norway) and humanely sacrificed (sharp blow to the head). Fish weights and lengths, and the presence of feces were registered and samples of skin were harvested. Skin for gene expression studies were sampled from two places, on the scaleless occipital part of the head (HS) and caudal of the dorsal fin (DF), a scaled area. Lice attachment is frequently observed at both places (Dawson et al., 1999; Grimnes and Jakobsen, 1996; Jaworski and Holm, 1992; Todd et al., 2000). Tissues were left in RNAlater (Ambion®, Austin, TX, USA) at 4 °C for 24 h before storage at 80 °C until further use. For histological examination, HS skin samples without scales from lice-infected and non-infected fish were harvested at 8 dpi, stored in 10% buffered formalin at 4 °C for 48 h, including a change of formalin after 24 h. Dehydration and paraffin embedding were performed by standard histological procedures. The embedding was performed with care to ensure that skin samples were transversely sectioned. After 5 weeks, the developmental stage and

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number of lice on the remaining fish in each tank were determined.

2.3. RNA isolation and cDNA synthesis

Total RNA extraction was performed with a combination of Trizol (Invitrogen, Thermo Fisher Scientific Inc, Carlsbad, CA, USA) and RNeasy Mini Kit (Qiagen, Hilden, Germany). Approximately 50 mg of tissue, Trizol and zirconium oxide beads were homogenized followed by adding chloroform and centrifugation to separate RNA into the supernatant. RNA cleanup was performed with 200 µl of the supernatant including on-column DNase digestion (Qiagen, Hilden, Germany) according to Qiagen protocol. Aliquots of 30 µl of RNAse free H_20 was used to elute RNA. The concentration of RNA was determined by spectrophotometry using NanoDrop ND1000 (Nanodrop Technologies, Wilmington, DE, USA). The RNA was stored at −80 °C until further use. The number of fish in each group was in HS: 17 from NI-C group, 20 from 4 dpi group and 15 from 8 dpi group. For DF: 14 from NI-C group, 20 from 4 dpi group and 13 from 8 dpi group.

For each skin sample 1600 ng of RNA was subjected to cDNA synthesis with a combination of random primers and oligo DT primers using the cDNA Affinity Script (Agilent Technologies, Matriks AS, Oslo, Norway) following the manufacturer's protocol. To exclude gDNA contamination in the following qPCR runs, samples without reverse transcriptase enzyme were also synthesized and included in each qPCR run. The synthesized cDNA was diluted 10 times by adding 180 µl of RNase free water and stored at −20 °C until further use.

2.4. qPCR protocol

Published gene sequences were used to design primers for quantitative Real Time PCR (qPCR) reactions by CLC Workbench software (Table 1). Previously published primer sequences were also used. Primers were designed so that all amplicon sizes fell within the range from 50 to approximately 200 base pairs in length.

qPCR experiments were performed on a LightCycler 96 Instrument (Roche diagnostics, Basel, Switzerland) 96-well plates and Light Cycler 480 SYBR Green I Master mix (Roche, Basel, Switzerland). For each gene, 4 µl of cDNA was run in duplicates from each fish, with the addition of specific primers at 10 µM concentration in a final volume of 12 µl. The qPCR cycle was as follows: initial 5 min denaturation step at 95 °C, followed by 40 cycles of denaturation (10 s at 95 °C), annealing (20 s at 60 °C) and extension (15 s at 72 °C). The cycling runs were all terminated by melting curve analysis where the fluorescence was measured during temperatures from 65 °C to 97 °C. For all genes, the maximum-second-derivative method (Roche Diagnostics) was used to calculate the crossing point value and the specificity of the PCR amplicon was confirmed by melting curve analysis and subsequent agarose gel electrophoresis. gDNA contamination was excluded by including wells with only H_20, primers and SybrGreen on each plate. PCR efficiency was calculated by two fold serial dilutions of cDNA for each primer pair in triplicates. Efficiencies ranged from 1.8 to 2.15.

The selection of reference genes for skin was based on previous studies performing gene expression normalization studies of Atlantic salmon tissues (Olsvik et al., 2005), including work on lice infected skin (Braden et al., 2015; Holm et al., 2015; Krasnov et al., 2012, 2015; Skugor et al., 2008; Tadiso et al., 2011). Among the most promising candidates were *elongation factor 1a* (*ef1a*), *eukaryotic translation initiation factor 3 subunit 6* (*eif3s6*) and *glyceraldehyde phosphate dehydrogenase* (*gapdh*), and *eif3s6* was found to have most stable Ct values across groups and time points.

2.5. Statistical analysis of qPCR data

The delta-delta CT method was used to calculate relative gene expression. Ct values were first normalized to the housekeeping gene *eif3s6*. Transformed values (ΔCt) were then compared to mean ΔCt values of all the NI-C fish to calculate the −ΔΔCt value. Statistical difference was determined for each gene between NI-C and infected groups of fish (4 and 8 dpi, respectively) by one-way ANOVA followed by Tukey's multiple comparisons test with a 5% probability level, if criteria for normality were met by the Shapiro-Wilk's test, and equality of variances by the Brown-Forsythe's test. If criteria for normality were not met, the Kruskal-Wallis test followed by Dunn's multiple comparisons test was used instead. Basal expression level difference between the two skin sites (in NI-C fish) was calculated by dividing the −ΔΔCt value in skin behind dorsal fin for each gene with the corresponding −ΔΔCt value found in skin on top of the head. Statistical difference at $P < 0.05$ level was calculated with the student's t-test if criteria for normality by the Shapiro-Wilkes test were met. If not, the Mann-Whitney test was used instead. The level of significance for all analyses was set at $P < 0.05$. GraphPad Prism version 6.0 (San Diego, CA, US) was used to make graphs and perform all statistical analyses.

2.6. Immunohistochemistry

The scaleless skin samples from the occipital part of the head collected at 8 dpi from infected and non-infected fish were processed for histology and immunohistochemistry (IHC). Embedded skin was sectioned at 3 µm width on SuperFrost Plus glass slides (Thermo Scientific, VWR, Radnor, PE, USA), prior to drying at 37 °C for at least 12 h before further processing. Parallel sections from two fish from each group were stained with Alcian blue-Periodic Acid Shift method following standard histological procedure.

The IHC reactions were performed at room temperature unless otherwise stated. Sections were incubated at 58 °C for 30 min, dewaxed in xylene and rehydrated in graded alcohol baths before transferring to distilled water. Sections were next autoclaved in 0.01 M citrate buffer, pH 6.0 at 120 °C for 10 min to retrieve antigens, followed by treatment with phenyl hydrazine (0.05%; Sigma-Aldrich, St. Louis, MO, USA) for 40 min at 37 °C to inhibit endogenous peroxidase. The slides were subsequently rinsed three times in phosphate-buffered saline (PBS). Nonspecific binding was prevented by adding normal goat serum diluted 1:50 in 5% BSA/TBS for 20 min. IHC was performed with the following antisera: anti-salmonid MHC class II, anti-salmonid Mx and anti-salmonid CD8α (Table 2). The polyclonal anti-salmonid Mx was diluted 1:4000 (kindly provided by Jo-Ann Leong, Hawaii Institute of Marine Biology, validated in (Xu et al., 2010)). Identification of MHC class II molecules was possible with the use of polyclonal antiserum (diluted 1: 600) made towards a recombinant protein of the salmon MCH class II β chain and positive cells have previously been identified in Atlantic salmon skin (Koppang et al., 2003). For CD8α (monoclonal), a 1:100 dilution was used (Hetland et al., 2010). The primary antibodies were all diluted in tris-buffered saline (TBS) with 1% BSA and incubated for 30 min (MHC class II, Mx) or overnight at 4 °C (CD8α) followed by rinsing three times in TBS, incubation with HRP labelled secondary antibody (EnVision© System kit; Dako, Glostrup, Denmark) for 30 min. To evoke the red color, sections were incubated with AEC for 15 min, followed by washing with distilled water, counterstaining with Mayer's hematoxylin for 1 min and mounted with polyvinyl alcohol media (PVA). Negative controls were performed using 1% BSA instead of the primary antibody.

Since the epidermal thickness can vary between fish (Holm et al., 2015), the area in which positive cells in the epidermis

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Table 1
Primer list. Primers used for gene expression analysis (qPCR).

Gene name and symbol	Accession	Size	Primers	Source
Arginase-2, mitochondrial (*argi2*)	BT058927.1	110	F:GACAGGCTCGGCATTCAGA R:AAAGACGGGTCCATCGCAT	(Holm et al., 2015; Kortner et al., 2012)
Cathelicidin antimicrobial peptide (*cath*)	AY360357.1	100	F:ACACCCTCAACACTGACC R:CCTCTTCTTGTCCGAATCTTCT	(Holm et al., 2015; Krasnov et al., 2015)
T-cell surface glycoprotein CD4 precursor (*cd4*)	BT056594	121	F:GAGTACACCTGCGCTGTGGAAT R:GGTTGACCTCCTGACCTACAAAGG	(Bakke-McKellep et al., 2007; Holm et al., 2015; Mikalsen et al., 2012; Xu et al., 2012)
Cluster of differentiation 8 α (*cd8α*)	AY693393	174	F:CACTGAGAGAGACGGAAGACG R:TTCAAAAACCTGCCATAAAGC	(Bakke-McKellep et al., 2007; Holm et al., 2015; Mikalsen et al., 2012; Xu et al., 2012)
CD83 antigen precursor (*cd83*)	BT047309	89	F:GCACCTGTAGGAGAGCAGAACC R:TCCCTTTCTTCTGATTGGTCTGT	(Haugland et al., 2012; Holm et al., 2015)
C type lectin receptor A (*ctl-a*)	NM_001123579.1	128	F:ATCCTGCACAGCAAGGAACAG R:TTGTCCACCCATCTCCAATCC	(Holm et al., 2015; Mutoloki et al., 2010)
Beta-defensin 1-like (*defb1l*)	EG781611	116	F:ATTTAGAAGACGTGGGCG R:GGATGCTCAAACTACAGTGG	(Krasnov et al., 2015)
Eukaryotic translation initiation factor 3 subunit 6 (*eif3s6*)	BT043738	92	F:GTCGCCGTACCAGCAGGTGATT R:CGTGGGCCATCTTCTTCTCGA	(Skugor et al., 2008)
Eomesodermin (*eomes*)	ACB87011	112	F:TGTGGGAAAGCAGACAACAAC R:GCTTCAGTTTGCCGAAGGAG	(Munang'andu et al., 2013)
Forkhead box P3 (*foxp3*)	HQ270469	65	F:AGCTGGCACAGCAGGAGTAT R:CGGGACAAGATCTGGGAGTA	(Munang'andu et al., 2013)
GATA-binding protein 3 (*gata3*)	EU418015	61	F:CCCAAGCGACGACTGTCT R:TCGTTTGACAGTTTGCACATGATG	(Munang'andu et al., 2013)
Hepcidin 1 (*hepc1*)	NM_001140849.1	125	F:TTCAGGTTCAAGCGTCAGAG R:AGGTCCTCAGAATTTGCAGC	(Sutherland et al., 2014)
Heme oxygenase (*hmox*)	XM_014180523.1	206	F:GTCAACGCATCACCCTTCTT R:ATGGGGTCCTTCATCCTCTT	This study
Interferon α (*ifnα*)	AY216594.1	163	F:TGGGAGGAGATATCACAAAGC R:TCCCAGGTGACAGATTTCAT	(Holm et al., 2015; Xu et al., 2012)
Interferon γ (*ifnγ*)	AY795563	159	F:CTAAAGAAGGACAACCGCAG R:CACCGTTAGAGGGAGAAATG	(Holm et al., 2015; Mikalsen et al., 2012; Xu et al., 2012)
Immunoglobulin M (*IgM*)	BT058539.1	66	F:TGAGGAGAACTGTGGGCTACACT R:TGTTAATGACCACTGAATGTGCAT	This study
Immunoglobulin T (*IgT*)	GQ907004.1	98	F:GGTGGTCATGGACGTACTATTT R: CCTGTGCAGGCTCATATCTT	This study
Interleukin 1 β (*il1β*)	XM_014170479.1	73	F:GCTGGAGAGTGCTGTGGAAGA R:TGCTTCCCTCCTGCTCGTAG	(Holm et al., 2015; Kvamme et al., 2013; Marjara et al., 2012)
Interleukin 4/13A (*il4/13a*)	NM_001204895.1	136	F:GACCACCACAAAATGCAAGGA R:GGTTGTCTTGGCTCTTCAC	(Holm et al., 2015; Krasnov et al., 2015)
Interleukin 8 (*il8*)	NM_001140710.2	136	F:ATTGAGACGGAAAGCAGACG R:CGCTGACATCCAGACAAATCT	(Holm et al., 2015)
Interleukin 17A (*il17a*)	GW574233	136	F:TGGTGTGTGCTGTGTGTCTATGC R:TTTCCCTCTGATTCCTCTGTGGG	(Marjara et al., 2012; Mutoloki et al., 2010)
Inducible nitric oxide synthase (*inos*)	AF088999	116	F:GGAGAGCCTTCTGGTTG R:ACCTTAACTTGTTCCTGAGATAC	(Holm et al., 2015; Mutoloki et al., 2010)
Leukocyte cell-derived chemotaxin 2 (*lect2*)	BT050009.2	89	F:TGTGGTGCTCATAGCTGT R:CTGTCCTCCTCCTGTTACT	(Holm et al., 2015)
Mannose binding protein C (*mbl2*)	XM_014194482	136	F:CCGAAGAACGAGGAGGAAA R:TGGAAAAGGTGAGGGGATG	This study
Major histocompatibility complex class II antigen (*mhc class II*)	XM_014191566.1	69	F:ATGGTGGAGCACATCAGCC R:CTCAGCCTCAGGCAGGGAC	(Haugland et al., 2005; Holm et al., 2015)
Myxovirus resistance 1 (*mx*)	U66475	78	F:TGCAACCACAGAGGCTTTGAA R:GGCTTGGTCAGGATGCCTAAT	(Holm et al., 2015; Svingerud et al., 2012; Xu et al., 2012)
Signal transducer and activator of transcription 1 alpha (*stat1a*)	GQ325309	127	F:AGTAAGGAGAGGGAGAAGG R:GCTTATCGTATTGGTCGTGT	This study
Signal transducer and activator of transcription 6 (*stat6*)	XM_014135765.1	185	F:TACTCTGACCCCCTCTCCAA R:TTCTTCCTACTCCTCCTCCTCC	This study
T-box 21 (*tbx21*)	HQ450583	57	F:AGTGAAGGAGGATGGTTCTGAG R:GGTGATGTCTGCGTTCTGATAG	(Holm et al., 2015; Munang'andu et al., 2013)
T-cell receptor γ (*tcrγ*)	EU221166	116	F:AGGCAGCAATCAACGAAAACC R:GCTTGACCAAGTCTGGAAACA	(Marjara et al., 2012)
Toll-like receptor 22A (*tlr22a*)	AM233509.1	149	F:GCCAATCTTACAGGACTCACAC R:CCACTCATAAAGTCATCATTCAAGG	This study
Transforming growth factor β-1 (*tgfβ*)	EU082211	191	F:AGTTGCCTTGTGATTGGGGA R:CTCTTCAGTAGTGGTTTGTCG	(Holm et al., 2015; Mutoloki et al., 2010)
Tumor necrosis factor α 1 + 2 (*tnfα*)	NM_001123589	173	F:AGGTTGGCTATGGAGGCTGT R:TCTGCTTCAATGTATGGTGGG	(Holm et al., 2015; Xu et al., 2012)

were counted was standardized before counting positive cells. The number of CD8α positive cells in the skin was counted in 3 epidermal areas of 30 000 μm^2 in two parallel sections from 5 fish in each group (non-infected and 8 dpi) at 200× magnification. Therefore, 30 areas were included in each group. The number of MHC class II positive cells was counted in 5 epidermal areas of 15 000 μm^2 at 400× magnification. Therefore, 25 areas were included in each group (non-infected and 8 dpi).

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Table 2
Antibody list.

Target	Antibody	Source	Concentration
MHC class II β chain (MHC class II)	MHC class II β chain	(Koppang et al., 2003)	1:600
CD8α	Sasa CD8 F1-29	(Hetland et al., 2010)	1:100
Mx	Rainbow trout Mx3	(Trobridge et al., 1997; Xu et al., 2010)	1:4000

All analyses were performed with a Zeiss Axioskop 2 (Oberkochen, Germany), and photographs were made with the Leica DFC420 (Leica microsystems, Wetzlar, Germany). Measurement of epidermis was performed with the Leica Image Analysis software. Number of positive cells in skin sections as detected by immunohistochemistry in non-infected fish and infected fish at 8 days post infection was analyzed by Welch's *t*-test.

3. Results

3.1. Lice numbers

Lice numbers were calculated at the end of the trial, when most lice had reached preadult 2 stages. The average number of lice was 15.7 ± 5 (n = 111), range 5–27.

3.2. Gene expression of skin

In non-infected fish, expression analysis from skin samples of HS and DF location showed (constitutive) gene expression levels that differed significantly between the two skin sites (Fig. 1). Moreover, a mix of genes involved in innate and adaptive immune defenses differed significantly in both locations. Most gene ratios (mean fold ± SEM) ranged from −2 to 2, and genes outside this range were *cluster of differentiation 8 α* (*cd8α*) (2.5 ± 0.4), *c type lectin receptor a* (*ctl-a*) (2.9 ± 0.3), immunoglobulins *IgM* and *IgT* (3.2 ± 0.2 and 6.2 ± 0.3, respectively), *beta-defensin 1-like* (*defb1l*) (4.6 ± 0.1) and *mannose binding protein C (mbl2)* (104 ± 0.1).

At 4 dpi in HS and DF locations, gene expression changed significantly compared to NI-C ($P < 0.05$). By 8 dpi HS and DF showed lesser differential gene regulation (Figs. 2–7). HS (compared to NI-C) showed 18 out of 32 genes differentially expressed at 4 dpi and 6 genes at 8 dpi. Similarly, DF (compared to NI-C) showed 13 differentially regulated genes at 4 dpi and 6 genes at 8 dpi. In detail, *interferon α* (*ifnα*) and *interferon γ* (*ifnγ*) were significantly upregulated with time at DF (Fig. 2). There was also a marked change ($P < 0.0001$) for *ifnγ* from −1.7 ± 0.22 ($P < 0.0001$) at 4 dpi to 0.06 ± 0.25 at 8 dpi in HS. The interferon inducible *myxovirus resistance 1* (*mx*) and Th1 and CD8 T cell transcription

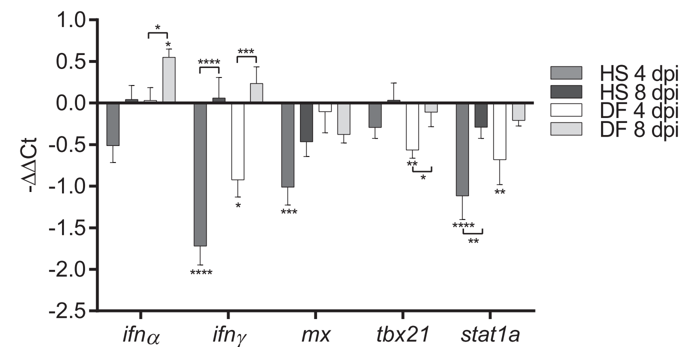

Fig. 2. Gene expression (qPCR) in skin from the top of the head (HS) and behind the dorsal fin (DF) in Atlantic salmon infected with *L. salmonis* at 4 and 8 dpi. Solid lines below or above asterisks represent significant changes between the denoted groups. Asterisks without line represent significant difference to non-infected control (NI-C). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ and ****$P < 0.0001$. Genes shown are *interferon α (ifnα), interferon γ (ifnγ),myxovirus resistance 1 (mx), T-box 21 (tbx21)* and *signal transducer and activator of transcription 1 alpha (stat1a).*

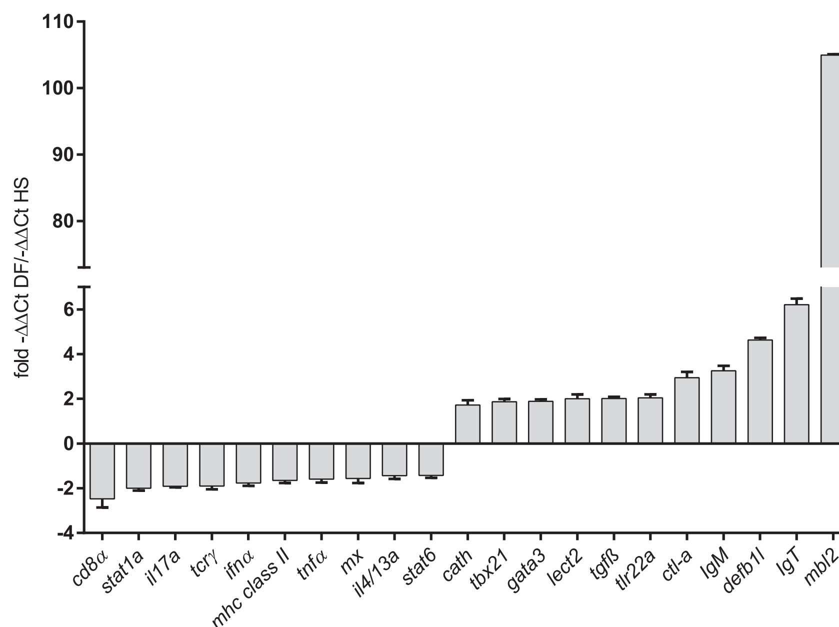

Fig. 1. Basal expression level fold ratio (fold -ΔΔCt DF/-ΔΔCt HS) between skin behind dorsal fin and skin on top of head of the genes *cd8α, stat1a, il17a, tcrγ, ifnα, mhc class II, tnfα, mx, il4/13a, stat6, cath, tbx21, gata3, lect2, tgfβ, tlr22a, ctl-a, IgM, defb1l, IgT* and *mbl2*. All genes showed a significant difference of at least $P < 0.05$.

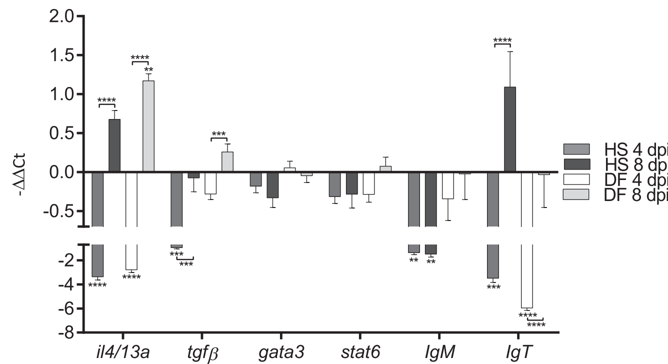

Fig. 3. Gene expression (qPCR) in skin from the top of the head (HS) and behind the dorsal fin (DF) in Atlantic salmon infected with *L. salmonis* at 4 and 8 dpi. Solid lines below or above asterisks represent significant changes between the denoted groups. Asterisks without line represent significant difference to non-infected control (NI-C). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ and ****$P < 0.0001$. Genes shown are *interleukin 4/13A* (*il4/13a*), *transforming growth factor β-1* (*tgfβ*), *GATA-binding protein 3 (gata3)*, *signal transducer and activator of transcription* 6 (*stat6*), *immunoglobulin M* (*IgM*) and *immunoglobulin T* (*IgT*).

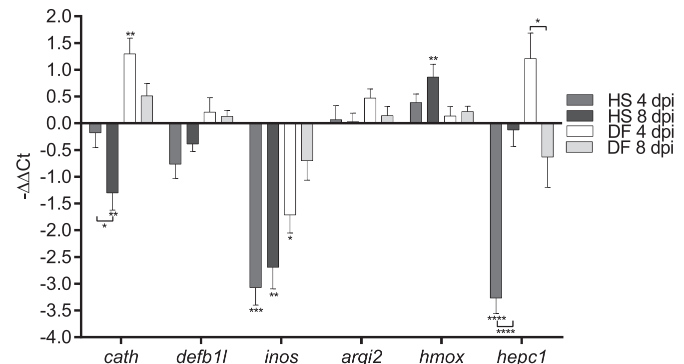

Fig. 6. Gene expression (qPCR) in skin from the top of the head (HS) and behind the dorsal fin (DF) in Atlantic salmon infected with *L. salmonis* at 4 and 8 dpi. Solid lines below or above asterisks represent significant changes between the denoted groups. Asterisks without line represent significant difference to non-infected control (NI-C). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ and ****$P < 0.0001$. Genes shown are *cathelicidin antimicrobial peptide* (*cath*), *beta-defensin 1-like (defb1l)*, *inducible nitric oxide synthase* (*inos*), *arginase* 2 (*argi2*), *heme oxygenase* (*hmox)* and *hepcidin 1* (*hepc1*).

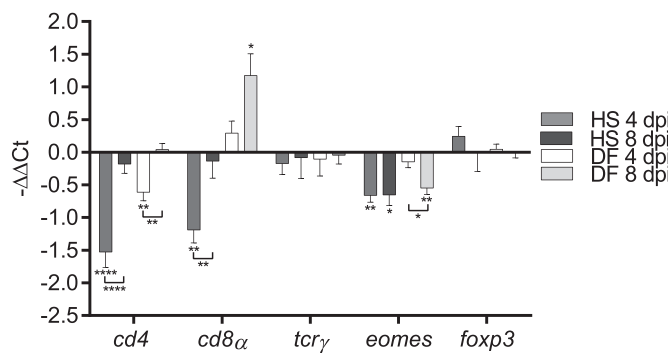

Fig. 4. Gene expression (qPCR) in skin from the top of the head (HS) and behind the dorsal fin (DF) in Atlantic salmon infected with *L. salmonis* at 4 and 8 dpi. Solid lines below or above asterisks represent significant changes between the denoted groups. Asterisks without line represent significant difference to non-infected control (NI-C). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ and ****$P < 0.0001$. Genes shown are *T-cell surface glycoprotein CD4 precursor* (*cd4*), *cluster of differentiation 8 alpha* (*cd8α*), *T-cell* receptor γ (*tcrγ*), *eomesodermin* (*eomes*) and *forkhead box P3* (*foxp3*).

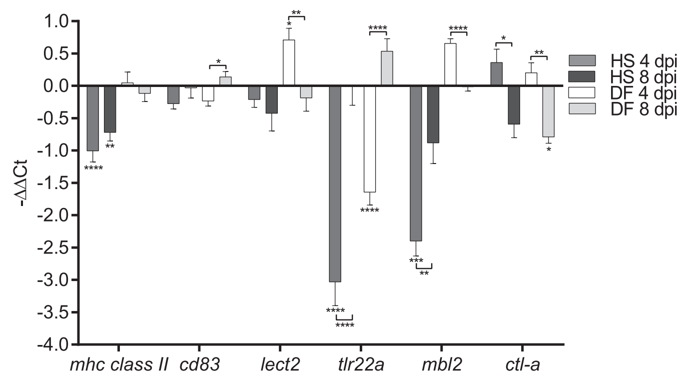

Fig. 7. Gene expression (qPCR) in skin from the top of the head (HS) and behind the dorsal fin (DF) in Atlantic salmon infected with *L. salmonis* at 4 and 8 dpi. Solid lines below asterisks represent significant changes between groups. Asterisks without line represent significant difference to non-infected control (NI-C). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ and ****$P < 0.0001$. Genes shown include *major histocompatibility complex class II antigen (mhc class II)*, *CD83 antigen precursor* (*cd83*), *leukocyte cell-derived chemotaxin 2* (*lect2*), *toll-like receptor 22A* (*tlr22a*), *mannose binding protein C (mbl2) and c type lectin receptor A* (*ctl-a*).

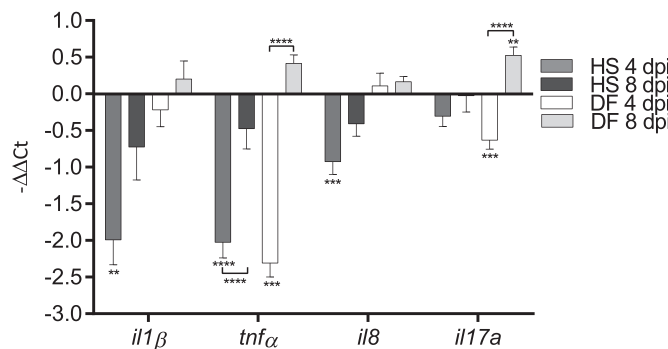

Fig. 5. Gene expression (qPCR) in skin from the top of the head (HS) and behind the dorsal fin (DF) in Atlantic salmon infected with *L. salmonis* at 4 and 8 dpi. Solid lines below or above asterisks represent significant changes between the denoted groups. Asterisks without line represent significant difference to non-infected control (NI-C). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ and ****$P < 0.0001$. Genes shown are *interleukin 1 β (il1β)*, *tumor necrosis factor α 1 + 2* (*tnfα*), *interleukin 8* (*il8*) and *interleukin 17A* (*il17a*).

factors *T-box 21* (*tbx21*) (Intlekofer et al., 2005; Lighvani et al., 2001; Sullivan et al., 2003; Szabo et al., 2000) and *signal transducer and activator of transcription 1 alpha* (*stat1a*) (Afkarian et al., 2002; Quigley et al., 2008) tended to increase over time. For HS (compared to NI-C), expression was significantly regulated at 4 dpi ($P < 0.001$, *mx, stat1a*) and with time ($P < 0.05$) in DF for *tbx21* and HS for *stat1a*.

Expression of Th2 associated markers were also temporally upregulated (Fig. 3). Th2 polarization inductor *interleukin 4/13A* (*il4/13a*) (Choi and Reiser, 1998; Takizawa et al., 2011) (Fig. 3) and *transforming growth factor β-1* (*tgfβ*) showed significant temporal upregulation at both skin locations, but marginal regulation of Th2 transcription factors *GATA-binding protein 3* (*gata3*) (Ho et al., 2009; Kanhere et al., 2012) and *signal transducer and activator of transcription 6* (*stat6*) (Zhu et al., 2001). The immunoglobulin *IgM* was significantly ($P < 0.01$) downregulated at both time points in HS. Lastly, the expression of the mucosal immunoglobulin *IgT* (Zhang et al., 2010) was significantly regulated ($P < 0.0001$) across time in HS and DF. For HS the mean of 3.5 ± 0.3 at 4 dpi changed to mean −ΔΔCt of 1.1 ± 0.5 at 8 dpi. Similarly, for DF; −5.9 ± 0.20 and −0.03 ± 0.4 at 4 and 8 dpi, respectively, compared to NI-C.

Temporal increased expression of T cell markers *T-cell surface glycoprotein CD4* (*cd4*) and *cd8α* was significant ($P < 0.01$) for HS, but only *cd8α* was significantly upregulated above NI-C level for DF

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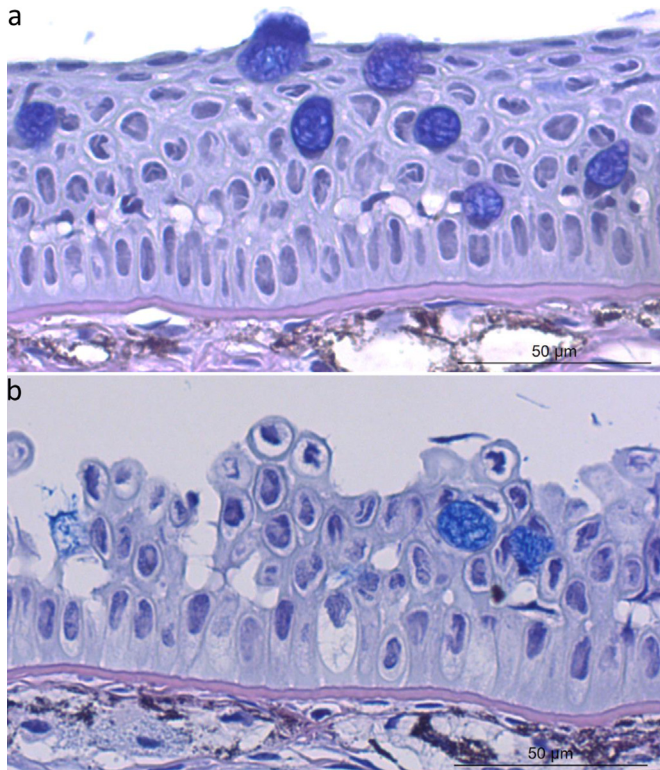

Fig. 8. Atlantic salmon epidermis from non-infected **(a)** and infected **(b)** with *L. salmonis* 8 dpi. AB-PAS stain.

($P < 0.05$; Fig. 4) at 8 dpi. *T-cell receptor* γ (*tcr*γ) showed minor regulations. Transcription factor for $CD8^+$ T cells *eomesodermin* (*eomes*) (Intlekofer et al., 2005) was significantly ($P < 0.05$) downregulated compared to NI-C at both time points in HS and DF except in DF at 4 dpi, and also temporally downregulated in DF ($P < 0.05$). *Forkhead box P3* (*foxp3*), transcription factor for regulatory T cells (Rudra et al., 2012), varied little compared to NI-C. Pro-inflammatory cytokines *interleukin 1* β (*il1*β), *tumor necrosis factor* α *1 + 2* (*tnf*α), *interleukin 8* (*il8*) and *interleukin 17A* (*il17a*) (Fig. 5) showed increased expression with time post challenge, exemplified by significant ($P < 0.0001$) increased expression of *tnf*α in HS and DF samples (Fig. 5). Expression of antimicrobial peptide (AMP) *cathelicidin antimicrobial peptide* (*cath*) differed in HS and DF samples (Fig. 6), and with time significant ($P < 0.05$) downregulation in HS. Little gene expression variation of *defb1l and arginase 2* (*argi2*) in comparison to NI-C was observed. *Inducible nitric oxide synthase* (*inos*) (Wiegertjes et al., 2016) on the other hand showed marked downregulation ($P < 0.05$) compared to NI-C in HS (-3.1 ± 0.3) and DF (-1.7 ± 0.3; Fig. 6) at 4 dpi. There was a slight increase in HS for *heme oxygenase* (*hmox*) ($P < 0.01$). Significant ($P < 0.0001$) responses for *hepcidin 1* (*hepc1*) was noteworthy in HS; mean $-\Delta\Delta$Ct level of -3.2 ± 0.3 at 4 dpi changed to a mean $-\Delta\Delta$Ct level of -0.12 ± 0.3 at 8 dpi.

Responses of *major histocompatibility complex class II* (*mhc class II*), *cd83 antigen precursor* (*cd83*) and *leukocyte derived chemotaxin 2* (*lect2*) were small but of note (Fig. 7). *Mhc class II* showed significant ($P < 0.01$) suppression at 4 and 8 dpi in HS (compared to NI-C), and *cd83* and *lect2* were significantly regulated in DF with time ($P < 0.05$). *Toll-like receptor 22a* (*tlr22a*), a pathogen recognizing receptor (Matsuo et al., 2008), was significantly upregulated with time in HS and DF ($P < 0.0001$) (Fig. 7). The $-\Delta\Delta$Ct level was -3.0 ± 0.4 ($P < 0.0001$) at 4 dpi and barely below zero at 8 dpi in HS. *Mbl2* showed significant temporal regulation in opposite directions in HS and DF, respectively. The $-\Delta\Delta$Ct level was -2.4 ± 0.2 ($P < 0.0001$) at 4 dpi and -0.9 ± 0.3 at 8 dpi in HS, but 0.65 ± 0.07 ($P < 0.0001$) at 4 dpi and zero at 8 dpi in HS. A small temporal decrease was also observed for *ctl-a*, significant ($P < 0.05$) at both HS and DF (Fig. 7).

3.3. Histochemistry and immunohistochemistry of epidermis

Changes in acidity and composition of mucus cells have been suggested to correlate with host responses to *L. salmonis* (Braden et al., 2015). Sections stained with Alcian blue-Periodic Acid Shift in this study could not identify differences in the acidity of mucus cells, both non-infected and infected skin sections consisted of mostly blue (acidic) mucus cells (Fig. 8a–b). However, the skin sections from infected fish showed signs of epithelial erosion (Fig. 8b).

IHC was applied to depict MHC class II^+, $CD8\alpha^+$ and Mx^+ cells in the scaleless skin samples. In CD8α stained sections (Figs. 9 and 10), positive cells were located above *stratum basale* and positive cells were typically found in clusters. The $CD8\alpha^+$ cells often had a morphology similar to lymphocytes (Fig. 9 c, 10 a-d). Counts of $CD8\alpha^+$ cells revealed a small, but significant ($P < 0.05$) increase in the number of positive cells in infected vs. non-infected fish. The number of positive cells ± SEM was 4.4 ± 0.56 in infected group, and 3.0 ± 0.33 in the control group.

Class II major histocompatibility (MHC class II) molecules are expressed on the surface of professional antigen-presenting cells (APC), including dendritic cells and macrophages. However, tissue

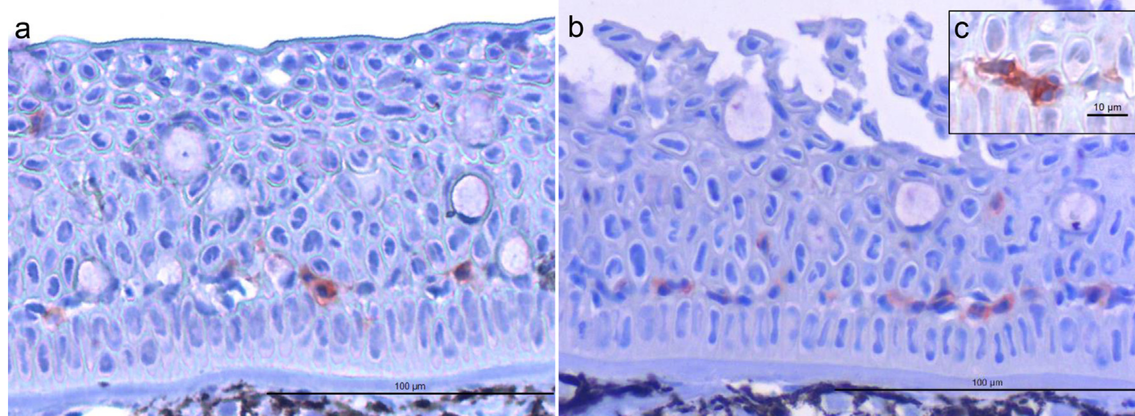

Fig. 9. a) $CD8\alpha^+$ cells in skin from non-infected fish. **b)** $CD8\alpha^+$ cells in skin from Atlantic salmon infected with *L. salmonis* at 8 dpi. **c)** Close-up view of the $CD8\alpha^+$ cells.

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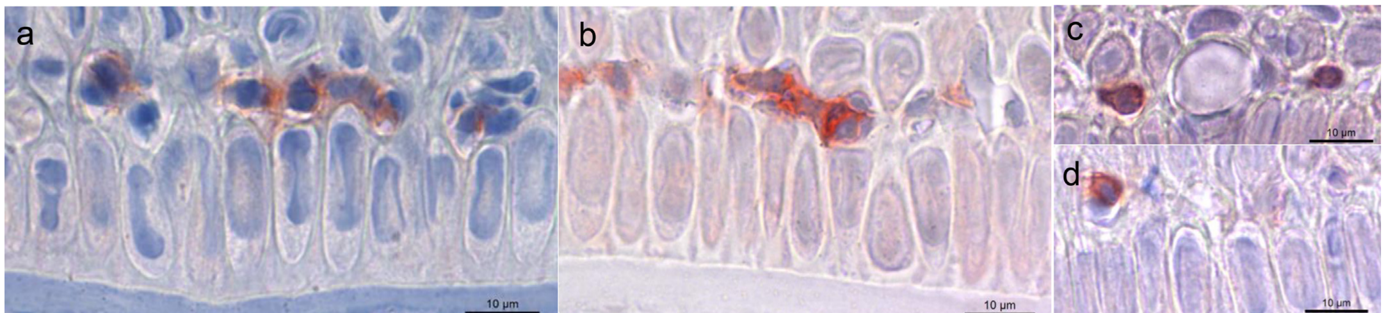

Fig. 10. a-d) CD8α+ cells in skin from Atlantic salmon infected with *L. salmonis* at 8 dpi.

parenchyma cells in mammals, including keratinocytes and other epithelial cells can also be positive (Gaspari et al., 1988; Holling et al., 2004). Positive reaction for MHC class II was distributed evenly in the epidermis (Fig. 11a,d). The positive cytoplasmic reaction was diffuse (Fig. 12a,c) as reported in (Koppang et al., 2003) but in addition also condensed (Fig. 12b,d) as described by (Braden et al., 2015). For the latter, a monoclonal antibody raised against MHC class II was used. Markedly, in this study, staining was observed close to the skin surface, in what appeared to be mature keratinocytes (Fig. 11a,d). No positive cells were seen in the dermis. Counts of strongly positive cells revealed no significant difference between infected and non-infected fish; 4.4 ± 0.43 (mean ± SEM) vs. 5.5 ± 0.02, respectively.

Most of the more strongly positive Mx cells were found in the top and the lower epidermal layers in both non-infected and infected fish (Fig. 13); cells in the middle layer of epidermis stained fainter (Fig. 13a–b). The positive cells found just above *stratum basale* showed strongest staining, some with a morphology similar to lymphocytes (Fig. 13d–e, 14a-b). Mx+ cells adjacent to the surface contained nuclei that was darkly stained (Fig. 13a, examples shown with arrowheads), or a large intensely stained cytoplasmic area (Fig. 13b, arrowheads). Most mucus cells were weakly positive (Fig. 13a). Melanin containing cells below the basal membrane were also faintly stained. A few sections including blood vessels in the dermis displayed endothelium with cells strongly positive for Mx (Fig. 13c).

4. Discussion

Here, we show results from the gene expression profiling of 32 genes performed in scaled and scaleless skin sampled at 4 and 8 dpi. In addition, three immune factors previously found to be responsive in Atlantic salmon exposed to *L. salmonis* at the gene expression level were investigated by IHC in scaleless skin. One striking finding was the difference in constitutive levels of a great number of immune-related genes between scaled skin versus scaleless skin (Fig. 1). A non-homogenous distribution of rainbow trout T cells was also reported in (Leal et al., 2016) between anterior and posterior parts of the body. In our study, the largest contrast in basal mRNA levels was observed for *mbl2* (104 fold difference), *IgT* (6.2 fold difference) and *defb1l* (4.6 fold difference), which showed a higher level of transcripts at DF compared to HS. All of these genes have previously been found responsive to lice; mannose binding lectins are commonly regulated during *L. salmonis* infections (Krasnov et al., 2012; Skugor et al., 2008; Sutherland et al., 2014); *IgT* is the most important mucosal antibody in fish (Zhang et al., 2010) upregulated early in skin and spleen of lice-challenged Atlantic salmon (Tadiso et al., 2011); and decreased expression of *defb1l* in relation to *cath* has been associated with reduced number of lice (Krasnov et al., 2015). The constitutive differences seen for those genes could affect the way fish respond to parasitic infections, and thus influence the spatial localization of parasites on the fish body. Fish ectoparasites have indeed been shown to have preferred sites of attachment (Jaworski and Holm, 1992; Lang et al., 1999; Lari et al., 2016; Todd et al., 2000). However, the significance of this finding in relation to the successful settlement of lice, attachment, further development, and finally, reproductive ability, merits further exploration.

An increase in transcript levels from 4 to 8 dpi was found for the majority of tested genes. This was in line with the expression of a

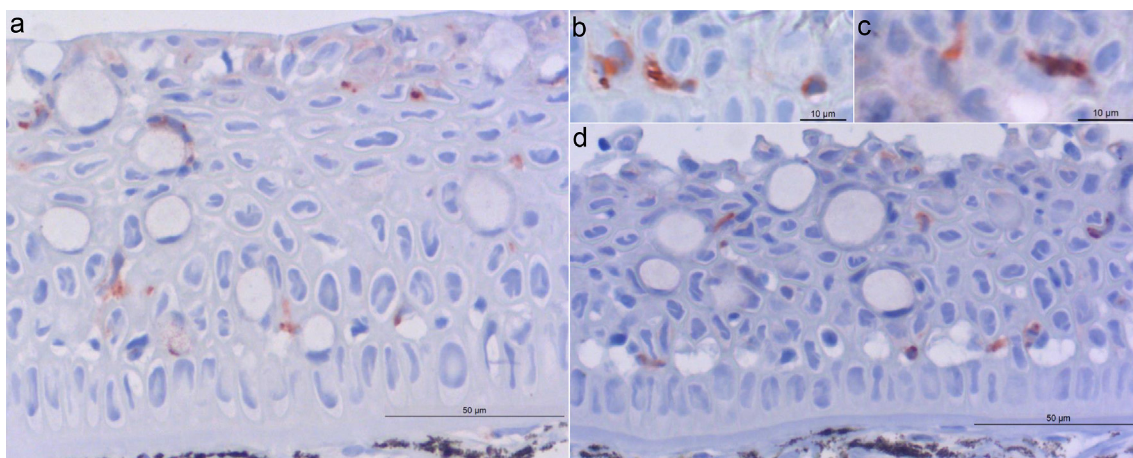

Fig. 11. a) MHC class II+ cells in skin from a non-infected Atlantic salmon. **b-d)** MHC class II+ cells in skin from Atlantic salmon infected with *L. salmonis* at 8 dpi.

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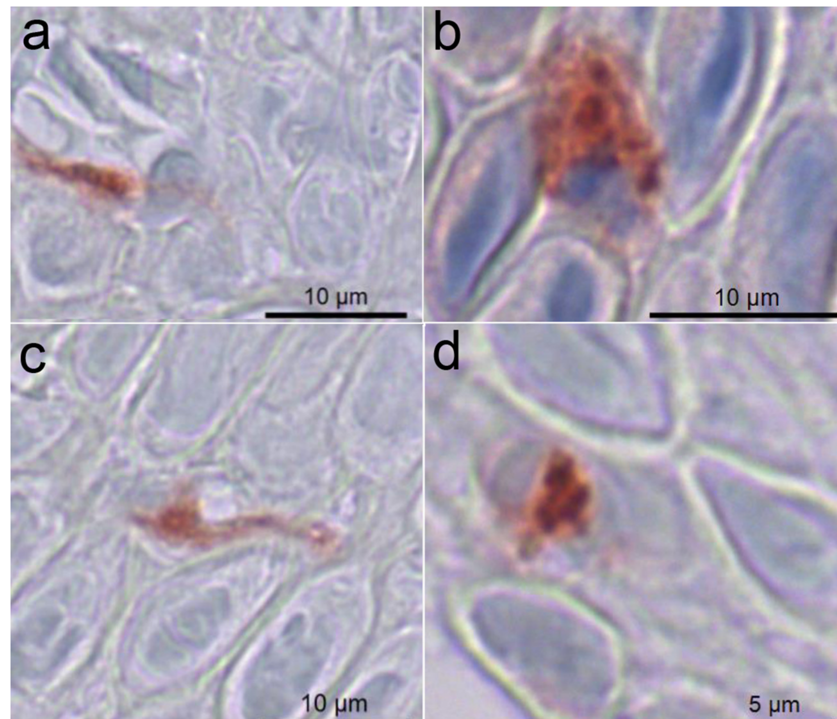

Fig. 12. a-d) MHC class II$^+$ cells in skin from Atlantic salmon infected with *L. salmonis* at 8 dpi.

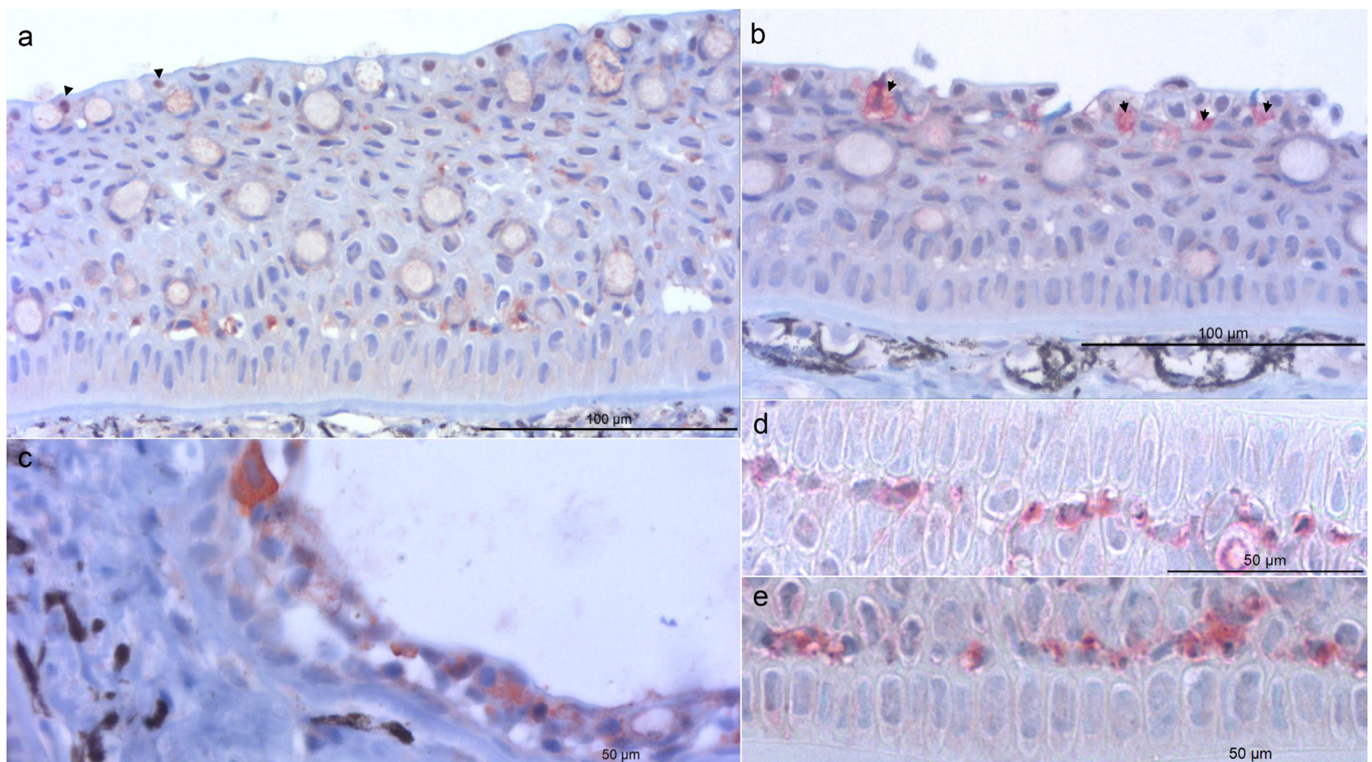

Fig. 13. a) Mx$^+$ cells from a non-infected Atlantic salmon. Arrowheads indicate positive reaction in nuclei **b**) Mx$^+$ cells in skin fish infected with *L. salmonis* at 8 dpi. Arrowheads indicate positive reaction in cytoplasm. **c**) Mx$^+$ cells in the endothelium of a fish infected with *L. salmonis* at 8 dpi. **d, e**) Close up view of the Mx$^+$ cells found apical of *stratum basale* in two Atlantic salmon infected with *L. salmonis* at 8 dpi.

cluster of immune genes, including a number of interferon-regulated genes, that showed similar biphasic responses (down-regulated from 1 to 5 dpi and then upregulated by 10 dpi) coinciding with the copepodid-chalimus transition (Tadiso et al., 2011). Interestingly, *hepc1*, an iron storage protein showed opposite regulation in DF and HS, with a temporal drop in expression in

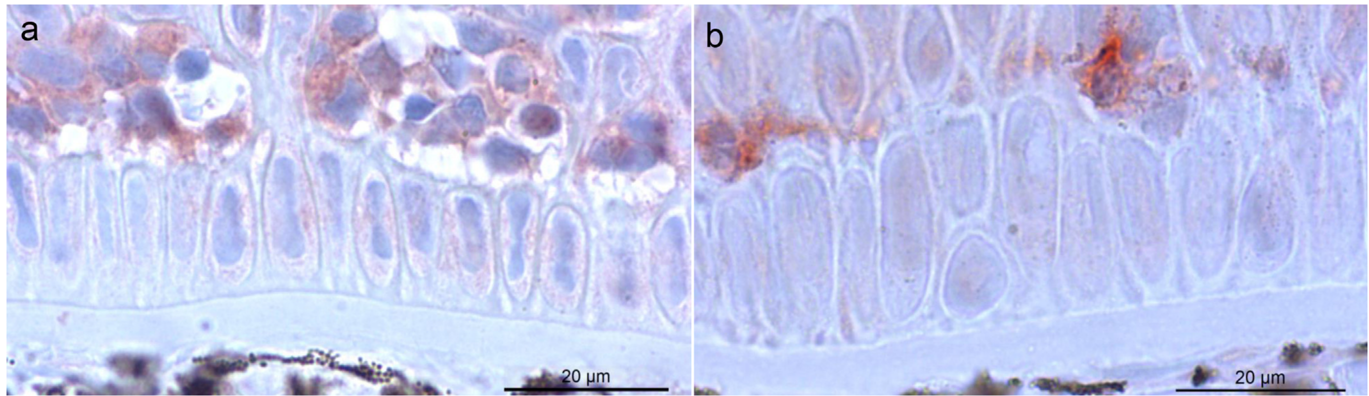

Fig. 14. a-b) Mx^+ cells in skin from Atlantic salmon infected with *L. salmonis* at 8 dpi.

DF (Fig. 6). The iron/heme withdrawal from tissues on which lice feed in response to *L. salmonis* attachment is considered an important mechanism of protection (Braden et al., 2015; Jodaa Holm et al., 2016; Sutherland et al., 2014). In line with this, *hmox* encoding heme oxygenase that degrades heme (Choi and Alam, 1996) and thus potentially makes it less available to lice showed higher levels in infected fish at both sites and at all times; however, the induction observed in infected fish appeared quite small.

Our previous studies have reported induction of a suite of anti-viral interferon related genes associated with the reduction in lice numbers at 3 dpi in fish fed sexual hormones (Krasnov et al., 2015), in fish that were bred selectively for increased lice resistance 3 weeks post infection (Holm et al., 2015), and in fish fed phytochemical-enriched functional feed 5 weeks post infection (Jodaa Holm et al., 2016). Fish possess TLR22 (Hu et al., 2015; Matsuo et al., 2008), which was suggested to have a similar role as TLR3, both functioning as dsRNA recognizing pattern receptors, inducing IFN and thus exerting a protective role in fish cells against dsRNA viruses (Matsuo et al., 2008). Levels of *tlr22a* mRNA were found to be significantly increased from 4 to 8 dpi at both skin sites (Fig. 7). Genes encoding IFN-inducible anti-viral effector protein *mx* was found upregulated in the lice-resistant salmon in all three aforementioned data sets (Holm et al., 2015; Jodaa Holm et al., 2016; Krasnov et al., 2015). *Mx* was found at significantly lower levels at 4 dpi in HS of infected compared to non-infected fish, while the difference disappeared at 8 dpi (Fig. 2) and was also not apparent by IHC. The importance of Mx regulation in salmon skin parasitized by lice is supported by findings of highly compartmentalized Mx tissue distribution in HS. (Figs. 13 and 14). Strongly Mx^+ cells were found apical to *stratum basale* (Fig. 14a–b). This was in the same location as most of $CD8\alpha^+$ cells (Fig. 10), implying that the area apical to *stratum basale* is of special importance, as possibly constitute an immunologically active type 1 immune compartment of the epidermis. We also showed Mx^+ cells in endothelium in dermis (Fig. 13c shows an example in one of the infected fish), in line with the similar finding of positively stained Mx^+ cells in salmon liver endothelium after *in vivo* injection of IFN plasmids (Chang et al., 2014). Type 1 interferons (IFNα and others) are often induced early after parasites invade the tissues in mammals, followed by a host-protective type 2 (IFNγ) interferon response (Beiting, 2014). This study found a temporal increase in the skin expression of *ifnα* (significant for DF) and *ifny* (significant for HS and DF) (Fig. 2); however the expression either slightly surpasses (*ifnα*) or is equal (*ifny*) to the non-infected control. *Eomes*, a CD8 transcription factor (Intlekofer et al., 2005), remained mainly downregulated at both time points (Fig. 4). The two other Type 1 transcription factors linked to CD8 T cell expansion, *tbx21* and *stat1a* (Intlekofer et al., 2005; Quigley et al., 2008), also showed lowest expression levels in skin at 4 dpi (Fig. 2). The *cd8α* transcript levels in HS at 8 dpi were significantly higher compared to 4 dpi while in DF at 8 dpi the expression significantly exceeded the non-infected control levels (Fig. 4). In line with this trend, IHC performed on HS tissue showed a small but significant rise in the number of $CD8\alpha^+$ cells. Similar to mammalian $CD8\alpha^+$ lymphocytes, these cells in salmon skin may be damaging to the parasite through anti-microbial granule-exocytosis dependent mechanisms (Colmenares et al., 2003; Cooper, 2009; Russell and Ley, 2002) or could contribute to the production of IFNy (Ghanekar et al., 2001; Uzonna et al., 2004), thus promoting downstream Type 1 effector responses. The tested MHC class II transcripts in HS showed lowest levels at 4 dpi compared to the non-infected control. The mRNA levels of MHC class II at 8 dpi still remained lower (Fig. 7). IHC also revealed slightly lower number of positive cells in infected fish; however, the difference was not significant. The distribution of MHC class II expression was seen throughout epidermis but notably, also in the apical epidermal region, implying that in addition to professional antigen presenting cells, keratinocytes, as the first line of contact with the parasite, might also be involved in MHC class II-mediated immune responses (Fig. 11a).

Th2-like polarization was previously reported in the susceptible Atlantic salmon while Th1/Th17 type of Th-guided responses was associated with responses to lice in resistant salmonids (Fast, 2014). An increase over time of several Th2-related factors occurred at both skin locations; canonical Th2 cytokine *il4/13a*, cellular differentiation regulator *tgfβ* that regulates Th2 polarization (Maeda and Shiraishi, 1996), and immunoglobulin *IgT*, likely a part of Th2-regulated effector responses (Fig. 3). *IgT* expression in infected fish compared to tissue-matched non-infected controls was stronger in HS than in DF (Fig. 3); possibly higher activation after infection in HS is needed since there is lower constitutive levels compared to DF (Fig. 1). Opposite regulation of *mbl2* from 4 to 8 dpi in HS (increase) and DF (decrease) compared to non-infected controls (Fig. 7) may follow the same logic as level of *mbl2* transcripts was much higher in DF compared to HS prior to infection (Fig. 1). Immune control of parasites could also involve temporal switches in the activation of different Th subsets (Moreau and Chauvin, 2010) and their spatial compartmentalization at different skin locations. Chettri et al. found expression of Th2 markers in skin of rainbow trout more prominent in later stages of infection with the ciliate *I. necator* (Chettri et al., 2014) while Braden et al. found Th1 markers upregulated during infection with adult lice stages followed by upregulation of markers of Th2 in lice resistant coho salmon (Braden et al., 2015). This study confirms previous findings (Holm et al., 2015; Jodaa Holm et al., 2016) that cytokines, their

receptors and other required molecules for both the Th1 and Th2 responses might be present at the same time in lice challenged Atlantic salmon. The outcome of such situation may be a mix of Th1/Th17 effector responses, likely involving IFN, CD8α and IL17 mediated responses, and Th2 effector responses that could result in increased IgT production and TGFβ-mediated tissue repair and wound healing.

5. Conclusions

In this study, we observed constitutive transcript level differences of immune genes in scaled and scaleless skin, preferred attachment sites for *L. salmonis*. During early time points of salmon louse infection, both tissue types showed increase in gene expression from 4 to 8 dpi for most studied immune related genes. The immunohistochemistry findings performed in HS at 8 dpi resulted in a number of relevant observations. Stronger staining of CD8α and Mx close to *stratum basale* in both infected and non-infected skin suggested compartmentalization of Type 1 protection mechanisms, and finally, increased number of cells expressing CD8α was observed in infected skin. MHC class II expression in the top epidermal layer warrants studies of keratinocytes' role in the initiation of skin immune responses. For a more complete picture, future studies should also address tissue and cellular distribution and dynamics of other relevant immune factors, including mediators of Type 2 responses.

Authors' contributions

HH, SS, AB and SW participated in the design of the study, conducted the experimental lice infection and prepared samples. HH and SR carried out the gene expression work. HH performed the immunohistochemistry with supervision by EOK. HH, SS, EOK and ØE interpreted the data. HH wrote the first draft of the manuscript. All authors participated in the active scientific discussion, edited, read and approved the final manuscript.

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Full length article

Difference in skin immune responses to infection with salmon louse (*Lepeophtheirus salmonis*) in Atlantic salmon (*Salmo salar* L.) of families selected for resistance and susceptibility

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ABSTRACT

Atlantic salmon is susceptible to the salmon louse (*Lepeophtheirus salmonis*) and the variation in susceptibility within the species can be exploited in selective breeding programs for louse resistant fish. In this study, lice counts were completed on 3000 siblings from 150 families of Atlantic salmon identified as high resistant (HR) and low resistant (LR) families in two independent challenge trials. Skin samples behind the dorsal fin (nearby lice attachment) were collected from ten extreme families (HR or LR) and analyzed by qPCR for the expression of 32 selected genes, including a number of genes involved in T helper cell (Th) mediated immune responses, which have been previously implied to play important roles during salmon louse infections.

Most genes showed lower expression patterns in the LR than in HR fish, suggesting an immunosuppressed state in LR families. The average number of lice (chalimi) was 9 in HR and 15 in LR fish. Large variation in lice counts was seen both within resistant and susceptible families, which enabled us to subdivide the groups into HR < 10 and HR > 10, and LR < 10 and LR > 10 to better understand the effect of lice burden per se. As expected, expression patterns were influenced both by genetic background and the number of attached parasites. Higher number of lice (>10) negatively affected gene expression in both HR and LR families. In general, strongest down-regulation was seen in LR > 10 and lesser down-regulation in HR < 10. HR in general and especially HR < 10 fish were better at resisting suppression of expression of both Th1 and Th2 genes. However, the best inverse correlation with infection level was seen for the prototypical Th1 genes, including several members from the interferon pathways. In addition, skin histomorphometry suggests that infected LR salmon had thicker epidermis in the area behind the dorsal fin and larger mucous cell size compared to infected HR fish, however marginally significant ($p = 0.08$). This histomorphometric finding was in line with the immune response being skewed in LR towards the Th2 rather than a Th1 profile. Our findings suggest that the ability to resist lice infection depends on the ability to avoid immunosuppression and not as much on the physical tissue barrier functions.

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1. Introduction

Infection with the salmon louse *Lepeophtheirus salmonis* is one of the major problems in the Atlantic salmon industry in the Northern hemisphere. Lice control is still largely dependent on the use of a limited number of pesticides and consequently, different and varied levels of resistance to different drugs have developed [1]. It is thus pertinent to develop novel biological methods of disease control. Although Atlantic salmon is considered particularly susceptible to *L. salmonis* compared to several other salmonid species [2–6], large variation in susceptibility to lice infection still exists. Glover et al. [7] reported genetically determined differences of infection levels in wild and farmed Norwegian Atlantic salmon. Heritability of resistance to lice was found to be moderate [0.33 ± 0.05] in a controlled infection trial by Gjerde et al. [8], and slightly lower by Kolstad et al. [9] [0.26 ± 0.07]. The level of observed heritability together with the large variance in phenotype

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suggests ample additive genetic variability, and thus a good potential for increasing the resistance through selective breeding.

Knowledge accumulated so far on immune responses to *L. salmonis* infection suggests that limited activation of T helper (Th) cell mediated immunity and a bias towards Th2 responses, define the susceptible Atlantic salmon phenotype, probably best classified as Th2-modified [10–12]. Differences in host responses between resistant and susceptible salmonid species also support the view that the character, timing and strength of the immune response play an important role for the outcome of the infection. Increase in the inflammatory cell infiltrate at the site of lice attachment, encapsulation of attached parasites by the hyperplastic response of keratinocytes and enhanced expression of pro-inflammatory genes from the Th1/Th17 pathways were linked to protection in several comparative interspecies studies [2,4,5,13]. Orally administered immunostimulants that promote Th1-type inflammatory responses have been shown to decrease lice burdens on Atlantic salmon in several challenge trials [14]. In addition to the number of established parasites, reduced ability to heal wounds has also been suggested as an additional problem that occurs during chronic infections in Atlantic salmon [11]. Suppression of multiple genes along the wound healing cascade, including down-regulation of structural proteins in parallel with the induction of extracellular matrix degrading metalloproteinases has been documented in several independent microarray studies [11,12,15]. It has been proposed that lice modulate the host immune responses by secretions that contain prostaglandin E2 and several proteases [16,17], which may explain some of these profiles. More detailed dissection of within-species protective mechanisms is still needed in Atlantic salmon. The main aim of the present study was to characterize differences in skin gene expression profiles between fish from high resistant (HR) and low resistant (LR) families, selected out of 150 Atlantic salmon families in two consecutive challenge trials. Gene expression correlates of protection were investigated among 32 candidate genes, including those involved in Th1, Th17 and Th2 mediated response. Multivariate statistics were applied to find correlations between lice number and the selected genes [18]. Finally, epidermal responses to infection were evaluated by measuring epidermal thickness and mucous cell size and number by light microscopy in selected HR and LR fish.

2. Methods

2.1. Challenge trials and fish

The selection of families and the trial were conducted as described [19]. In brief, the fish used in this study were pedigree fish from the 10th generation of the Aquagen Atlantic salmon (*Salmo salar* L.) breeding nucleus. In early November 2010, 255 females and 230 males were crossed, resulting in 491 families, first fed in February 2011, and individually tagged (Pit-tags) at 5–10 g in May 2011. In October 2011, 6000 seawater-adapted smolts (~60 g) from 150 families were transferred to NOFIMA AS research station at Averøy, Norway. The group was divided in two replicates where all 150 families were equally represented, and both groups where challenged with lice by the same protocol but at a different time. The first group was challenged with sea lice in July 2012. The challenge was performed as previously described [8,19], using closed tarpaulins with a total of approximately 50 copepodites per fish. The sea lice was allowed to develop until the chalimus II stage [20] before the individual weight, length and sea lice count were registered for each fish. In total, data from 2850 fish of an average weight of 800 g were registered in the July 2012 challenge (Trial 1). The second challenge was performed in October 2012 (Trial 2), and data was collected from 2348 fish with an average weight of 1900 g. Based on the results from Test 1, ten families where selected for sampling in challenge test 2; five families in each of the high resistant (HR) and low resistant (LR) groups.

2.2. Statistical analysis of challenge trial results

Density of attached lice was calculated by the formula number of lice/$\sqrt[3]{\text{body weight}^2}$.

The lice density was analyzed using univariate animal models, with the following general characteristics:

$$\mathbf{y} = \mathbf{X}b + \mathbf{Z}a + \mathbf{e}$$

where $\mathbf{y}$ is a vector of phenotypes (lice density), $\mathbf{a} \sim \mathrm{N}(0, \mathbf{G}\sigma_g^2)$ is a vector of random additive genetic effects, where $\mathbf{G}$ is a given relationship matrix (model dependent), and $\mathbf{e} \sim \mathrm{N}(0, \mathbf{I}\sigma_e^2)$ is a vector of random residuals. The fixed effects (**b**) included person (responsible for counting) by day. From this model the breeding values were estimated (EBV), which were then used to rank families into high resistant (HR) and low resistant (LR) family groups.

2.3. Sample collection

At 3 weeks post-infection in Trial 2, skin was sampled from 18 to 19 individuals from LR and HR families, respectively. In addition, 11 uninfected control fish from a nearby pen that belonged neither to LR nor HR families (ordinary background) were sacrificed for skin sample collection. Skin samples for gene expression analysis were excised behind the dorsal fin, an area where lice attachment was frequently observed. For the histological analysis, 6 fish from LR and HR groups and 5 fish from the uninfected control were selected.

2.4. RNA isolation and cDNA synthesis

Total RNA extraction was performed with a combination of Trizol (GIBCOL, Life Technologies) and RNAeasy Mini Kit (Qiagen). Approximately 50 mg of tissue, 1 ml Trizol and 1.4 mm zirconium oxide beads (VWR) were added to each sample, and homogenized in FastPrep-24 homogenizer (MP Biomedicals) at 6.5 × *g* for 5 min twice with incubation on ice for 5 min in between the runs of homogenization. After incubation for 5 min at room temperature, 0.2 ml of chloroform was added, and the samples were vortexed for 15 s, then incubated at room temperature for 3 min followed by centrifugation at 10,000× *g* for 15 min at 4 °C to separate RNA into the supernatant. 200 µl of the supernatant was subjected to RNA cleanup with 15 min of on-column DNase digestion according to Qiagen protocol. Aliquot of 30 µl of RNAse free H_2O was used to elute RNA. The concentration of RNA was determined by spectrophotometry using NanoDrop ND1000 (Nanodrop Technologies). The RNA was stored at −80 °C until further use.

For each sample, 3000 ng was subjected to cDNA synthesis using the cDNA Affinity Script (Agilent Technologies) following the manufacturer's protocol. For each reaction, 1 µl of random primers and 2 µl of oligo DT primers were used. Genomic DNA contamination was excluded by performing qPCR reactions using isolated RNA from selected samples as templates together with primers for elongation factor-1 α (EF-1a). The synthesized cDNA was diluted 10 times by adding 180 µl of RNase free water and stored at −20 °C until further use.

2.5. qPCR protocol

Published gene sequences were used to design primers for quantitative Real Time PCR (qPCR) reactions by CLC Workbench

software (Table 1). Primers were designed so that all amplicon sizes fall within the range from 50 to maximum 200 base pairs in length. qPCR experiments were performed on a LightCycler 480 Instrument (Roche, Applied Science) by using 96-well plates and Light Cycler 480 SYBR Green I Master mix (Roche). For each gene, 4 µl of cDNA was run in duplicates from each fish, with the addition of specific primers at 10 µM concentration in a final volume of 12 µl. The qPCR cycle was as follows: initial 5 min denaturation step at

Table 1
Different oligonucleotide primers used in the real-time PCR setup.

Real-time primers			
Gene name and symbol	Genbank	Efficiency	Primers
Arginase 2 (ARG2)	BT058927	1992	F:GACAGGCCGGCATTCAG R:AAAGACGGGTCCATCGCAT
Barrier to autointegration factor 1 (BANF)	BT049316.2	1920	F:ACAGACCCCTCATCATCCTG R:CGGTGCTTTTGAGAAGTGGT
Cathelicidin-derived antimicrobial peptide 2 (CATH)	AY542961.1	1897	F:CCTCTTCTTGTCCGAATCTTCT R:ACACCCTCAACACTGACC
CD209 type lectin A (CTL-A)	BT046430.2	1954	F:ATCCTGCACAGCAAGGAACAG R:TTGTCCACCCATCTCCAATCC
CD4	BT056594	1734	F:GAGTACACCTGCGCTGTGGAAT R:GGTTGACCTCCTGACCTACAAAGG
CD83	BT047309.2	2	F:GCACCTGTAGGAGAGCAGAACC R:TCCCTTTCTTCTGATTGGTCTGT
Complement C3A (C3A)	L24433.1	1900	F:GAGGAAAGGTGAGCCAGATG R:TGTGTGTGTCGTCAGCTTCG
CXCL10	AJ417078.1	1858	F:CAGGTGGGTCATTCTAAAGC R:CTTGGCAAATGGAGCTTCTG
Cyclooxygenase 2 (COX2)	AY848944.1	1948	F:CCTATCTTACTACACACGGACG R:TTGGCATCTGGGAGCAC
Elongation factor 1 alpha (EF1-α)	BT072490.1	2	F:GCTGTGCGTGACATGAGG R:ACTTTGTGACCTTGCCGC
GATA-binding protein 3 (GATA3)	NM_001171800.1	1866	F:AGCTGAAAGACAACCAAACTG R:CATAAAGCCTGTCTCCCCAC
Lymphocyte G0/G1 switch protein 2 (GOS2)	BT049510.1	1875	F:TCCATTCGCTAAGGAGATGC R:TAGAACCCAGCAGGTACACCT T
Inducible nitric oxide synthase (iNOS)	AF088999.1	1956	F:GGAGAGCCTTCTGGTTG R:ACCTTAACTTGTTCCTGAGATAC
Interferon stimulated gene 15 (ISG15)	NM_001123640.1	1741	F:AAGTGATGGTGCTGATTACGG R:TTGGCTTTGAACTGGGTTACA
Interferon α (IFNα)	DQ354155.1	2	F:TGGGAGGAGATATCACAAAGC R:TCCCAGGTGACAGATTTCAT
Interferon γ (IFNγ)	FJ263446/AY795563	1886	F:CTAAAGAAGGACAACCGCAG CACCGTTAGAGGGAGAAATG
Interleukin 11 (IL11)	AJ535687	2	F:TCAACTCCCTTGAGATGAGACC R:TCCTGGGAAGACTGTAACACATC
Interleukin 4/13 (IL4/13)	AB574339	1861	F:GACCACCACAAAATGCAAGGA R:GGTTGTCTTGGCTCTTCAC
Interleukin 17D (IL17D)	EU689087.1	2	F:CTTGTCTCCCTGGGTATGAACCT R:CAATATGCCTCGGGTATGAACCT
Interleukin 1 beta (IL1β)	NM_001123582.1	1859	F:GCTGGAGAGTGCTGTGGAAGA R:TGCTTCCCTCCTGCTCGTAG
Interleukin 8 (IL8)	NM_001140710.2	2	F:ATTGAGACGGAAAGCAGACG R:CGCTGACATCCAGACAAATCT
Keratin type II cytoskeletal 8 (K8)	BT059558	2	F:CCAGCCACAACACTCCAA ??? R:TATCCACCGCCATATCCACC
Leukocyte cell-derived chemotaxin 2 (LECT2)	BT050009.2	1883	F:TGTGGTGCTCATAGCTGT R:CTGTCCTCCTCCTGTTACT
Matrix metalloproteinase 13 (MMP13)	NM_001140524.1	1928	F:TTGTTCAGGGCTGCTTCTT R:CCAGAAGACAGTCCGTGTG
MHC-II	BT060311.1	2	F:ATGGTGGAGCACATCAGCC R:CTCAGCCTCAGGCAGGGAC
MX	NM_001139918.1/BT044881	1827	F:TGCAACCACAGAGGCTTTGAA R:GGCTTGGTCAGGATGCCTAAT
Myeloperoxidase (MPO)	BT072012.1	1996	F:TGCCATGTTCCCCAATGT R:CGATACCACCCTCAAAAACT
PDL1 (Programmed cell death 1 ligand 1)	NM_001141351.1	2	F:TCAACGACTCTGGGGTGTACCGATG R:TCCACCTCATCTCCACCACGTCTC
Proliferative cell nuclear antigen (PCNA)	BT056931	1995	F:TGAGCTCGTCGGGTATCTCT R:GTCCTCATTCCCAGCACACT
T-bet	HQ450583	1928	F:AGTGAAGGAGGATGGTTCTGAG R:GGTGATGTCTGCGTTCTGATAG
Tissue growth factor beta (TGFβ)	EU082211	2	F:AGTTGCCTTGTGATTGGGGA R:CTCTTCAGTAGTGGTTTGTCG
Tumor necrosis factor 1 + 2 (TNFα)	NM_001123589	1968	F:AGGTTGGCTATGGAGGCTGT R:TCTGCTTCAATGTATGGTGGG
Zymogen granule 16 (ZG16)	BT057545.1	1988	F:GTTGAGGTGTCTGGGAAGT R:GTTGGCTGGGTAGAAGTTG

95 °C, followed by 40 cycles of denaturation (10 s at 95 °C), annealing (20 s at 60 °C) and extension (15 s at 72 °C). The cycling runs were all terminated by melting curve analysis where the fluorescence was measured during temperatures from 65 °C to 97 °C. The maximum-second-derivative method (Roche diagnostics) was used to calculate the crossing point value and the specificity of the PCR amplicon was confirmed by melting curve analysis and subsequent agarose gel electrophoresis. PCR efficiencies were calculated from two fold serial dilutions of cDNA for each primer pair in triplicates.

2.6. Statistical analysis of qPCR data

The delta–delta CT method was used to calculate relative gene expression. Ct values of HR, LR and uninfected controls were first normalized to the housekeeping gene EF-1a. Transformed HR and LR values (ΔCt) were then compared to mean ΔCt values of the uninfected control fish of ordinary background to calculate −ΔΔCt. Amplification efficiencies for all genes were similar (see primer Table 1), which allowed us to show results as −ΔΔCt values. Statistical analysis was performed using the software GraphPad Prism version 6.0. One-way ANOVA followed by Dunnett's multiple comparisons test were executed between each of the four groups (HR > 10, HR < 10, LR > 10, LR > 10) and uninfected control.

Multivariate analysis that included Principal component analysis (PCA) and Partial Least Squares Regression (PLRS) was performed by using The Unscrambler (version 10.1, CAMO Process AS, Oslo, Norway) and in-house developed routines written in MATLAB (version R2010a, The MathWorks, Natick, MA).

PCA is a powerful multivariate method often used for analyzing large data sets. It is especially useful for finding the most important variables in the system through calculating the latent variables or the principal components (PC). On the basis of PCA results the differences between the analyzed samples (fish) can be visualized through score plots. Furthermore, the causes of sample differentiation can be analyzed through correlation loading plots, where measured variables (genes) are plotted in accordance with their influence (significance) on the variation in the system and in accordance with their mutual relations (correlation coefficients) [18,21]. In this study the PCA was used as an exploratory step and results are not presented here.

PLSR is a multivariate regression method that analyses the variation in *X* data table by employing the PCA and predicts the variation in the *Y* data table. The PLRS analysis was used to test the ability of predicting the number of lice (*Y* block) by gene expression −ΔΔCt values (*X* block) [18,21,22]. Fish were divided in four groups by resistance (HR or LR) and within each, according to the number of lice they were infected with and as such were projected onto the correlation loading plot (they were defined as category variables that were not used in calculating the PLSR model). This was done in order to better understand the relation between the degree of lice infection and the gene expression.

2.7. Histopathology

Dehydration, paraffin-embedding, sectioning and staining with periodic acid-Schiff (PAS) were done according to standard histological procedures. The 2 μm sections were examined with a Leica DFC 420 microscope. The embedding was done carefully to make sure that samples were transversely sectioned.

2.8. Quantification of epidermal thickness and mucous cell density

The epidermis on the PAS-stained blinded sections was subjected to morphometric analysis by using the Leica Image Analysis software, following in part method described earlier [23]. Three representative/selected areas (SA) of the outermost epidermis were chosen for quantification. First, a 200 μm polygonal line was drawn along the outer surface of the epidermis, and then two straight lines were drawn from the beginning and the end of this line perpendicular to the basement membrane. The mean of these two straight lines were calculated to find the epidermal thickness for this SA. In each of the polygons, the area occupied by mucous cells was calculated by outlining the circumference of each mucous cell. If cells were overlapping, the total area of the cells was calculated. The density of mucous cells was calculated by adding together each of the mucous cells. This number was thereafter divided by the area of the SA in order to find the ratio between mucous cells and epidermis, and thereby also the percentage of epidermis that is occupied by mucous cells.

The number of mucous cells, mean epidermal thickness and density of mucous cells were calculated for each SA. Mean values for each fish was then calculated, fish were divided into LR and HR groups, and students *t*-test was calculated between the groups (LR and HR) and the control for each place of skin (dorsal fin and sideline). The *p*-value calculated when comparing best statistically significant difference achieved was $p < 0.08$.

3. Results

3.1. Estimation of heritabilities, breeding values and family ranking

Lice count in Trial 1 was on average (for both HR and LR groups) 21.0 while in Trial 2, the average number was 12.8 lice per fish. LR fish selected for analysis in this study from Trial 2 had on average (mean ± SD) 14.7 ± 5.6 lice/fish and HR fish 9.5 ± 9.6 lice/fish. The genetic correlation between the two challenge tests was estimated to be 0.64 for the lice count and 0.55 for the density of lice. The heritabilities for the two tests were estimated [19] and were low to moderate; 0.14 ± 0.03 and 0.13 ± 0.03 for Trial 1 and 2, respectively. The estimated genetic correlation between lice resistance in the two tests was high (0.72 ± 0.12) [19]. EBV (estimated breeding values) calculated on the basis of lice density in Trial 1 and 2 were used to rank families and select the ten most extreme HR and LR families. Separation of families according to EBV is shown (Fig. 1).

3.2. Gene expression

All sampled fish from HR and LR families were included in the gene expression analysis. The lice number ranged from 3 to 36 in the LR fish and from 2 to 24 in HR fish. Subdivision of fish according

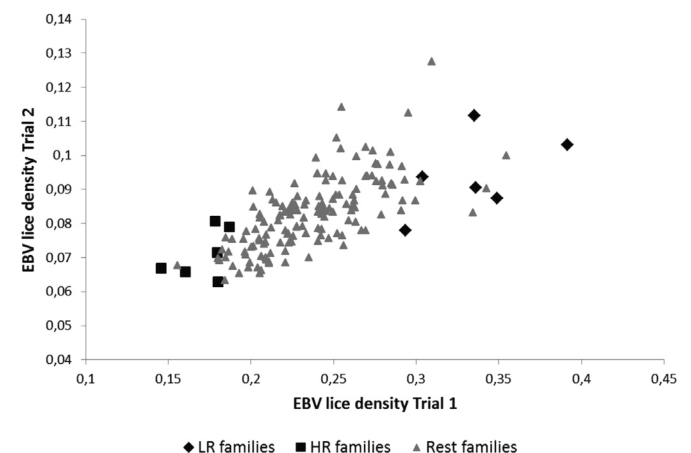

Fig. 1. The correlation between mean family breeding value (EBV = estimated breeding value) of the different lice challenged families in Trial 1 and Trial 2 ($r^2 = 0.7$).

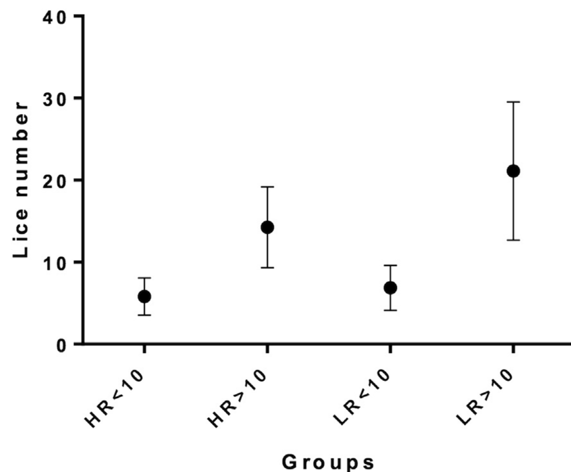

Fig. 2. Lice numbers shown as mean ± SD in the two HR and two LR groups. Number of individual fish in each group: HR > 10 = 8, HR < 10 = 11, LR < 10 = 8, LR > 10 = 10.

to the number of lice (more or less than 10) into HR < 10, HR > 10 and LR < 10 and LR > 10 enabled us to separate family background effects from the effect of lice burden on gene expression. Mean number of lice in four groups is shown in Fig. 2. Functions of the 32 target genes are shown in Table 2. A number of genes represent functional pathways involved in innate immunity as well as Th1 and Th2 type of immune responses that were previously found to be differentially regulated following lice attachment and also genes from the interferon pathways. Most of the genes were down-regulated in HR and LR groups compared to uninfected control, in line with the general immunosuppressed state observed during chalimus infection [11,12]. Expression patterns of individual genes are shown in Fig. 3. Overall, magnitude of measured gene expression responses was small. ARG2, a representative of Th2 immune responses, showed the highest level of down-regulation of genes tested (Fig. 3A). In LR < 10 group, it had the mean −ΔΔCt value of −4.6, a 24-fold down-regulation compared to uninfected control. On the other side of the spectrum, CD209, a representative of type 2 responses, showed the highest induction (Fig. 3D). It had −ΔΔCt value of 2 in HR < 10, equal to a 4-fold increase compared to uninfected control. Furthermore, differences in gene expression between the four groups were found to be small but systematic, as found by multivariate analysis. Generated multivariate models accounted for a significant portion of gene response variation and assisted in the biological interpretation of data.

PCA was used as the first explorative tool and it outlined a separation between HR and LR groups. This separation was already visible along the first principal component, which accounts for 27% of the variation in the analyzed data (plots not shown). This finding shows that the difference between HR and LR groups is the dominating variation in the analyzed system. The preliminary findings obtained by PCA were used for defining the follow-up PLSR analysis, by which the number of lice and the four groups (**Y**) were predicted by the gene expression data (**X**). Results of the PLSR analysis are presented by score plot in which the differences between the analyzed fish is outlined (Fig. 4), and by correlation loading plot by which the underlying causes for the observed differences are determined (on the basis of the correlation coefficients between the measured variables and the calculated principal components) (Fig. 5). In brief, position of a variable in the correlation loading plot designates its significance for the calculated PLSR model (the closer to the outer circle that depict correlation coefficient equal to 1, the more significant the variable), and its relation to other variables. If

Table 2
Gene function overview showing the main functional characteristics of genes examined by real time PCR.

Arginase 2 (ARG2)
- found in alternatively activated macrophages, as part of Th2 response; competing with nitric oxide (NO) synthesis by using the same substrate (L-arginine); activated in skin of louse-infected Atlantic salmon [11,38,39]

Barrier to autointegration factor 1 (BANF)
- involved in chromatin organization; represses gene expression; involved in early response to viruses in liver, heart and ASK cells in Atlantic salmon; correlated with IFNα expression [15,40,41]

Cathelicidin-derived antimicrobial peptide 2 (CATH)
- immunomodulatory and antimicrobial peptide, inhibiting IFNγ induced Th1 responses; induced by microbial stimuli in Atlantic salmon cell lines; early induction by lice infection in spleen of Atlantic salmon [12,42,43]

C type lectin A (CD209, DC-SIGN)
- pathogen recognizing receptor on dendritic cells; IL4 dependent expression; mediates Th2 activation in zebrafish; early expression in louse-infected Atlantic salmon skin [12,44–46]

CD4
- marker of T helper cells, biphasic activation in louse-infected Atlantic salmon skin [11,12,47]

CD83
- marker of antigen presenting cells in mammals; regulation of T- and B-cell maturation and responses; present on mononuclear cells and TØ cell line in Atlantic salmon [48–51]

Complement C3A (C3A)
- shown to activate classical, lectin and alternative complement pathways in zebrafish; produced mainly in liver; widespread extrahepatic distribution, including skin in Atlantic salmon [52,53]

CXCL10
- involved in chemotaxis of leukocytes, regulator of IFN and Th1 responses induced by IFNγ and CpG DNA in Atlantic salmon cells [54–56]

Cyclooxygenase 2 (COX2)
- mediates formation of prostaglandins, which are also found in lice secretory/excretory products; up-regulated in head kidney of louse-infected Atlantic salmon [16,57–59]

GATA-binding protein 3 (GATA3)
- key transcription factor that controls polarization of Th2 cells in mammals; expressed at high levels in thymus, gill, and skin, in concert with the Th2 cytokine IL4/13A in salmonids [30,60,61]

Lymphocyte G0/G1 switch protein 2 (GOS2)
- Proapoptotic, induced by TNFα; increased transcription in skin of louse-infected salmon [11,62]

Inducible nitric oxide synthase (iNOS)
- produced in IL1β, IFNγ and TNFα stimulated mammalian macrophages; responsive to a number of parasitic infections in mammals and salmonids; early up regulation in louse-infected Atlantic salmon skin [2,63–65]

Interferon α (IFNα)
- part of Th1 response; induces antiviral state in cells; increased expression in response to several viruses in Atlantic salmon, especially alphaviruses [66,67]

Interferon stimulated gene 15 (ISG15)
- activated by IFN; involved in intracellular and extracellular antimicrobial defence; induced in IFN and dsRNA stimulated leukocytes [68–71]

Interferon gamma (IFNγ)
- part of Th1 response; defence against viruses and intracellular bacteria; identified in salmonids; increases pro-inflammatory cellular and gene expression responses [72–76]

Interleukin 4/13 (IL4/13)
- induces Th2 polarization; constitutively expressed in salmonid skin [30,77]

Interleukin 11 (IL11)
- induces Th2 polarization; appears to be part of Th2 response in Atlantic salmon [78,79]

Interleukin 17D (IL17D)
- stimulates inflammation in mammals; skin expression in Atlantic salmon associated with bacterial infection [80,81]

Interleukin 1 beta (IL1β)
- involved in Th1 and Th17 differentiation and acute phase response in mammals; strongly expressed in keratinocytes; increased expression in skin in response to bacteria and parasites in Atlantic salmon; highly responsive to *L. salmonis* infection in skin and immune organs [2,14,37,82–86]

Interleukin 8 (IL8)
- chemokine that attracts neutrophils; detected in salmon head kidney cells; up-regulated by LPS and *Piscirickettsia salmonis*; responds to *L. salmonis* attachment in skin [2,26,27]

Table 2 (*continued*)

Keratin type II cytoskeletal 8 (K8)
- intermediate filament protein, also involved in the contractile function; provides resilience against physical trauma [87,88]
Leukocyte cell-derived chemotaxin 2 (LECT2)
- neutrophil attractant; highly induced in *Aeromonas salmonicida* and *Staphylococcus aureus* infection in zebrafish; induced at sites of lice attachment [11,44,89]
Matrix metalloproteinase 13 (MMP13)
- collagenase that remodels extracellular matrix; associated with immune, stress and/or wound healing response during *L. salmonis* infection [2,10–12,90]
Major histocompatibility class II (MHC-II)
- surface marker of antigen presenting cells; reduced expression in Atlantic salmon head kidney cells after incubation with PGE2 and LPS; regulated in skin and internal organs during the infection with *L. salmonis* [2,11,12,57,91]
MX
- IFN induced; antiviral activity against a wide range of viruses in fish and mammals [71,73,74,92]
Programmed cell death 1 ligand 1 (PDL1)
- inhibitor of T helper cell activation, protect tissues from immunopathology up regulated in CMS affected Atlantic salmon hearts, could be involved in anergy of T helper cells upon lice attachment in skin [11,40,93–95]
Proliferative cell nuclear antigen (PCNA)
- involved in DNA synthesis, damage repair and cell cycle control; associated with stress response in Atlantic salmon skin [96–98]
T-bet
- transcription factor that controls polarization of Th1 cells in mammals; negatively correlated with increase in antibody levels in salmon, implying the Th1 role [29,61,99]
Tissue growth factor beta (TGFβ)
- regulator of immune responses, cellular proliferation and differentiation, remodeling and wound healing; up-regulated by lice-induced damage in skin [11,100]
Tumor necrosis factor alpha (TNFα)
- major orchestrator of Th1 response; up-regulated in response to viruses and bacteria in Atlantic salmon, more highly expressed in salmonid species more resistant to *L. salmonis* [2,71,82,101]
Zymogen granule (ZG16)
- likely involved in secretory pathways; regulated during lice infections [102]

two variables are on the opposite sides of the axes of the correlation loading plot then they are negatively correlated.

In the score plot presented in Fig. 4, fish are labeled in accordance to the resistance to lice low (LR) and high (HR), where the extremes (<10 and >10 lice) are also used for grouping (Fig. 4). As can be seen, on the basis of gene expression data fish can be well separated based on difference in lice number and family origin. Generally, the higher the lice count, the more immunosuppressive effect could be observed for most of the genes. The strongest suppression was seen in LR fish with LR > 10 fish showing lowest expression levels for most genes. Less than 10 attached lice on LR fish alleviate the suppression to a large extent for a fair number of genes. For the most part, however, salmon from the HR < 10 group showed higher gene activity than the LR < 10 group, as illustrated by TNFα, iNOS and ARG2 (Fig. 3A) IFNy, MX and ISG15 (Fig. 3B), as well as CXCL10, CD83 and TGFβ (Fig. 3C), revealing a strong influence of the fish genetic background.

In the correlation loading plot of the PLSR analysis presented in Fig. 5, majority of the analyzed genes are negatively correlated to lice counts and LR > 10 group of fish. This means that the higher the number of lice, the lower the expression of these genes. In the correlation loading plot it is seen that the genes that are positively correlated to each other cluster in 4 groups: group A consists mainly of Th1 inflammatory markers, group B includes most of Th2 related genes, group D with the smallest number of genes that characterize LR < 10 fish, and group C in the middle of the plot with a minor contribution to the model.

Th1 inflammatory markers: TNFα, iNOS, IFNα and BANF (Fig. 3A), IFNγ, MX and ISG15 (Fig. 3B), CXCL10 and CD83 (Fig. 3C), as well as T helper cell marker CD4, clustered together in ellipse A in the outer left part of the correlation loading plot (Fig. 5). All were highly negatively correlated to lice count and LR > 10 while being highly positively correlated to HR < 10 group of fish. C3A and TGFβ (Fig. 3C) are also found in ellipse A; although it seems less likely they play a role in Th1 responses. Key markers of Th2 immunity (CD209, LECT2, IL4/13 and IL11) clustered together in ellipse B (Fig. 5, individual gene expression presented in Fig. 3D). The cluster also included IL8, PCNA, MMP13 and K8 with roles in chemotaxis of neutrophil cells, cellular proliferation, extracellular remodeling and strengthening of the skin physical barrier. CATH, an antimicrobial protein likely exerting multiple immunomodulatory functions (Fig. 3D), was also found in ellipse B within the outer ring in the upper left quadrant. This cluster was negatively correlated to the lice count and LR > 10, however, less so than cluster A. Also, most genes from cluster B stand in moderate anticorrelation to most genes from cluster A, supporting the polarization of Th1 and Th2 responses. Expression patterns of MHC-II, GOS2, COX2 and ZG16 (Fig. 3F) were associated with protection achieved in the LR < 10 group of fish. Genes encircled in cluster C are found in the lower part of the correlation loading plot and were negatively correlated to lice count and LR > 10, but also to HR < 10. The group of genes from cluster D is found situated close to the center of the correlation loading plot (intersection of the first and second principal component) and are therefore not significant for the obtained PLSR model. As can be seen in Fig. 3G showing individual gene expressions, most of the genes responded to infection almost equally in all four groups, including, somewhat surprisingly, transcription factors T-bet and GATA3, crucially involved in the polarization of activated T-cells into Th1 and Th2 direction, respectively. Genes from cluster D were highly correlated with the HR > 10 group.

3.3. Histological results

In the selected fish, the number of lice were 16 ± 9 in LR and 10 ± 5 in HR (mean ± SD). All parameters were highest in sideline from the LR fish; thickness of epidermis and number of mucous cells in LR fish were significant at $p < 0.08$, in contrast to the uninfected control fish with the lowest values overall. See Table 3 for results. Although HR fish had the mean largest mucous cell size along the sideline ($p < 0.08$), this result was not reflected in the density of mucous cells. Here, LR fish remained having significantly highest density of mucous cells. Parameters related to mucous cells were also lowest in sideline sampled from uninfected control fish.

4. Discussion

Different salmonid species exhibit varying susceptibility to salmon louse infection, which implies there is a genetic basis for selection of more resistant individuals through breeding. Findings from the present and several previous studies show that there is ample additive genetic variability within Atlantic salmon families [8,9]. However, laborious and time consuming counting of lice on thousands of breeding candidates is currently the only method of assessing resistance in a population. It is expected that screening of whole genomes will in due time provide genomic markers associated with increased resistance to lice and that molecular breeding would complement breeding approaches that require quantification of the phenotype. Interestingly, genome-wide association studies in mammals revealed that great majority of significant SNP-phenotype associations are found in noncoding genomic regions, which apparently alter transcription [24]. Establishing links between regulatory DNA variation and salmon

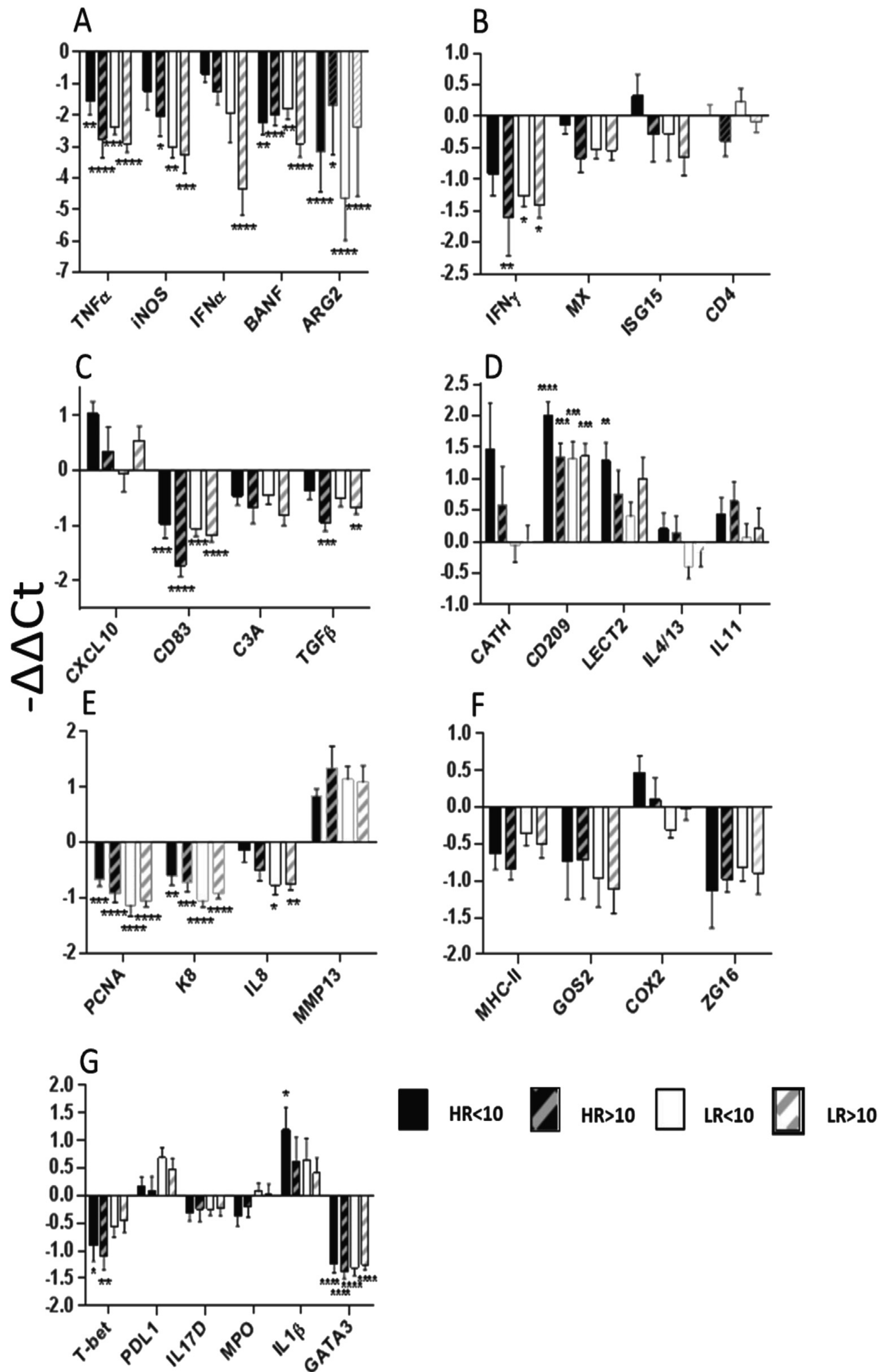

Fig. 3. Relative gene expression in skin behind dorsal fin from *L. salmonis*-infected HR and LR fish and further sub-grouped by lice number (more or less than 10). Relative gene expression is presented as $-\Delta\Delta Ct$. Bars represent mean ± SEM compared to uninfected control fish. Number of fish in each group: HR > 10 = 8, HR < 10 = 11, LR < 10 = 8, LR > 10 = 10. One way ANOVA followed by Post hoc Dunnet's multiple comparisons test were performed between groups and control. Asterisks denote significant difference between groups and control: **** = $p < 0.0001$, *** = $p < 0.001$, ** = $p < 0.01$, * = $p < 0.05$. A) Gene expression of tumor necrosis factor alpha (TNFα), inducible nitric oxide synthase (iNOS), interferon alpha (IFNα), barrier-to-autointegration factor 1 (BANF) and arginase 2 (ARG2). B) Relative gene expression of interferon gamma (IFNγ), MX, interferon stimulating gene 15 (ISG15) and CD4. C) Relative gene expression of CXCL10, CD83, complement C3 (C3A) and tissue growth factor beta (TGFβ). D) Relative gene expression of cathelicidin (CATH), CD209, leukocyte cell derived chemotaxin 2 (LECT2), interleukin 4/13 (IL4/13) and interleukin 11 (IL11). E) Relative gene expression of proliferative cell nuclear antigen (PCNA), keratin type II cytoskeletal 8 (K8), interleukin 8 (IL8) and matrix metalloproteinase 13 (MMP13). F) Relative gene expression of major histocompatibility class II (MHC-II), lymphocyte G0/G1 switch protein 2 (GOS2), cyclooxygenase 2 (COX2) and zymogen granule 8 (ZG16). G) Relative gene expression of T-bet, programmed cell death ligand 1 (PDL1), interleukin 17D (IL17D), myeloperoxidase (MPO), interleukin 1 beta (IL1β) and GATA-binding protein 3 (GATA3).

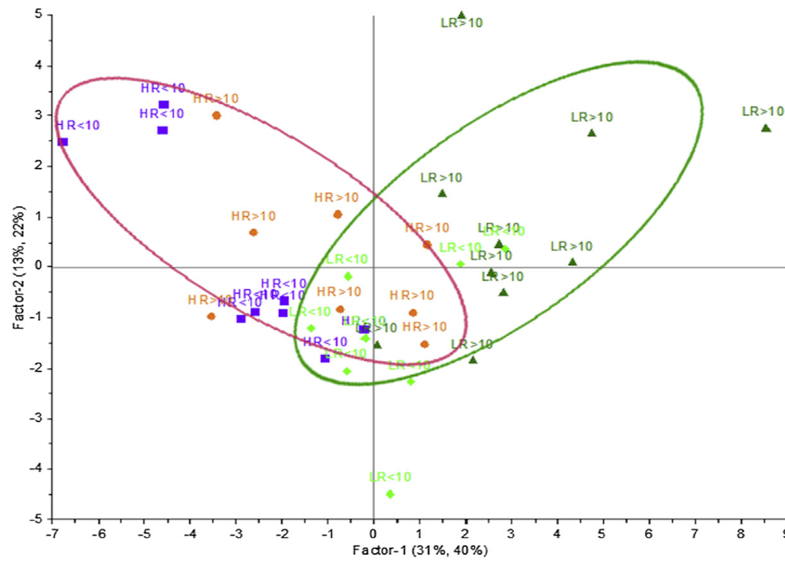

Fig. 4. PLRS score plot that presents the relation between samples, i.e. fish labeled in accordance with their resistance profile; low (LR) and high (HR) and lice number (<10 and >10 lice). LR and HR fish are separated on the basis of the gene expression data. The extreme families, HR < 10 and LR > 10 show the highest separation (farthest apart) in accordance with gene expression profiles being indicative of resistance category. Variables on opposite side of origin have negative correlation. For more details of the PLSR method see Methods section.

transcriptional responses to lice is likely the future in this field. One of the main features of resistant fish revealed in this study was lesser down-regulation of majority of examined genes compared to susceptible fish thus suggesting that differences in the magnitude of transcriptional responses may be the key to resistance to the parasite.

Large number of screened families and individuals per family allowed for subgrouping of fish according to the number of

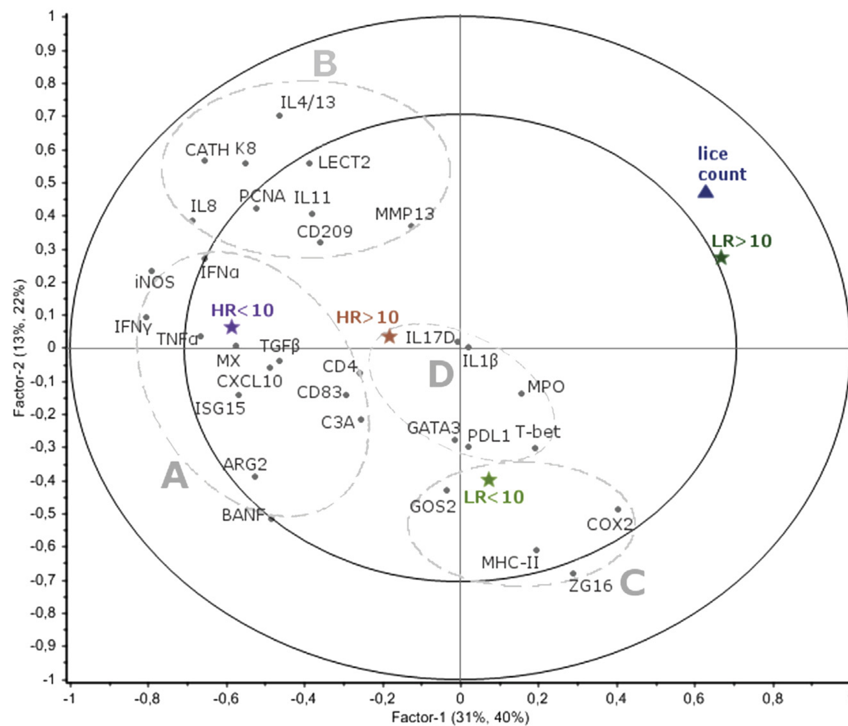

Fig. 5. PLSR correlation loading plot presented to explain relationship among variables: genes (*X*), presented in gray and lice count (*Y*). Fish groups are presented in color (HR > or <10 and LR > 10 or <10) and were projected onto the plot. Ellipses A, B, C and D outline the clusters of genes (variables) that grouped together and that are correlated. Ellipses A, B and C that encircle the genes plotted close to the outer circle of the correlation loading plot, depict genes that are significant for the obtained PLSR model. Genes in ellipses A and B are also highly positively correlated, whilst negatively correlated to genes in ellipse C. Ellipse D that encircles the genes plotted closer to the center of the correlation loading plot (intersection of PCs) designate the genes that are not significant for the obtained PLSR model. As can be seen HR < 10 fish relate to ellipse A and is also on the same side as ellipse B, indicating a relation between sea lice infection and the variables (genes) in this group of fish. For more details of the PLSR method see Methods section. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 3
Number and size of mucous cells, epidermal thickness and percentage of epidermis comprising mucous cells in fish from low resistant families (LR), high resistant families (HR) and control fish (C), respectively. Values were determined using three selected areas (SA) on skin sections sampled from behind dorsal fin and from the sideline. Number (No.) of mucous cells = the number of mucous cells. n = number of fish examined from this group. SA = selected area. Number of SA examined (total number of SA examined in each group).

	Group	No. of mucous cells (mean ± SD)	Mucous cell size (μm) (mean ± SD)	Mucous cell density (% of SA)	Thickness of epidermis (μm) (mean ± SD)	n	No. of SA examined
Skin behind the dorsal fin	LR	10.44 ± 4.54*	239.1 ± 78.5	18.40	82.14 ± 17*	6	18
	HR	8.85 ± 5	218.15 ± 54	14.50	70.73 ± 14	6	18
	C	8.90 ± 4.39	194.37 ± 45.5	13.34	66.06 ± 20	5	15
Skin along the sideline	LR	9.22 ± 3.35	226.77 ± 42.2	17.67*	63.15 ± 15	6	18
	HR	7.77 ± 2.9	252.10 ± 54*	14.53	74.62 ± 13	6	18
	C	7.4 ± 4.10	190.96 ± 62	11.17	70.25 ± 20	5	15

*T-test, $p < 0.08$.

parasites in addition to classification based on their genetic background. This enabled us to understand better which genes are the most influenced by the genetic background of the fish. Of those we found IFNα, as well as iNOS, a reactive nitrogen species producing enzyme in macrophages [25], and IL8, an attractant for neutrophil cells [26,27]. These genes had higher activity in both of the HR groups (HR < 10; HR > 10) compared to two LR groups.

Previously, Gjerde et al. [8] showed that ranking of Atlantic salmon families according to their resistance to lice was conserved in consecutive trials (including the same families) although individuals within those families differed in lice counts from trial to trial. Our findings suggest that individual lice counts vary within families and one can question if lice count is the most precise proxy of resistance. The expression of a handful of immune genes in this study, not negatively affected by increasing number of lice, might reflect more precisely an individual's potential to resist infection. The expression of macrophage and neutrophil markers (iNOS, IL8, LECT2 and CD83) supports previous literature that linked lice resistance to the inflammatory cell influx at the site of infection [5]. On the other hand, lice load clearly affected the expression of numerous other genes, e.g. IFNα was significantly down-regulated in LR fish with more than 10 lice. Classical Th1-mediated or type 1 responses are characterized by the expression of cytokines TNFα and IFNγ [28] and transcription factor T-bet [29] while Th2 responses are driven by the type 2 cytokines IL4 and IL13, encoded by one gene in teleosts, IL4/13 [30]. Th2 markers also include IL-11 and receptor CD209. Wound healing is associated with down-regulation of Th2 cytokines, including IL11, while proinflammatory type 1 mediators, such as TNFα and IFNγ are associated with accelerated healing response [31].

Multivariate statistical analysis found that most genes from ellipse B stand in moderate anti-correlation to most genes from ellipse A (Fig. 5), in support of a polarization of Th11 and Th2 responses. Th1 and 2 immune responses are however not mutually exclusive to the extent believed earlier [32] and we found both of them anti-correlated to the lice number. There is a possibility that their coexistence is required for the expression of the resistant phenotype. Multivariate statistical analysis identified IFNα, IFNγ, BANF, iNOS, CATH, K8, IL8 and IL4/13 as potential markers of resistance. This was determined by their placement in the outer ring of the correlation plot which explains 50–100% of the data variance (Fig. 5), and their negative correlation to lice count and LR > 10. Most of these genes were in fact down-regulated in comparison to uninfected control, and the multivariate statistical analysis outlined them as most anti-correlated to lice number and fairly influential. Lesser activity of a crucial Th2 molecule ARG2 in HR fish may have also contributed to the resistant HR phenotype. However, a time course study designed to investigate the expression dynamics of involved immune genes, especially those involved in Th1 and Th2 mediated immunity, should be pursued in future studies.

Skin histomorphological analysis revealed differences between infected individuals from the HR and LR families. The area behind the dorsal fin is one of the preferred attachment areas for *L. salmonis* during the later developmental stages while skin overlaying the sideline is typically free of lice. Higher values of all studied parameters in infected fish compared to uninfected control, and especially in the LR fish, confirmed skin responsiveness at the site of parasite attachment. Others have previously reported differences in epidermal parameters among resistant and susceptible salmonids. In two interspecies comparative studies [4,33], a thicker epidermis was observed in rainbow trout and Atlantic salmon compared to coho salmon. The fact that both species are more susceptible to lice made it difficult to draw a clear conclusion as to the relevance of skin thickness in relation to lice susceptibility. Within species comparison in our study was more informative. Our conclusion was that 20% thicker epidermis in LR compared to the uninfected control probably had minor impact on parasite numbers/density as HR fish was not significantly different from control. Furthermore, the number of mucous cells was higher behind the dorsal fin in LR fish ($p \approx 0.08$); however the influence of different lice burden in LR and HR groups must be taken into account. Dislodging and expelling resident parasites by mucous hypersecretion and smooth muscle hyper-contractibility are part of the Th2 host response [34,35]. Increased production of mucous has previously been described in Atlantic salmon following *L. salmonis* infection [4] and in salmon, mucous cell density has been found correlated with *Gyrodactylus salaris* infection intensity [36]. Our findings suggest that increased production of mucous is of limited protective value against lice infection. On the contrary, increased mucous production by Atlantic salmon might be important for parasite survival, serving as a nutritional component of the diet of developing lice, as has been discussed for *Gyrodactylus* infection [37].

Our findings are indicative of HR fish having a better ability to resist sea lice-mediated immunosuppression than LR fish, as well as skewing immunity towards Th1 responses. The search for genome markers for marker assisted selection should include exploration of non-coding DNA, with particular attention to sequences involved in the regulation of immune gene transcription.

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III

Jodaa Holm *et al. Parasites & Vectors* (2016) 9:271
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RESEARCH Open Access

Dietary phytochemicals modulate skin gene expression profiles and result in reduced lice counts after experimental infection in Atlantic salmon

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Abstract

Background: The use of phytochemicals is a promising solution in biological control against salmon louse (*Lepeophtheirus salmonis*). Glucosinolates belong to a diverse group of compounds used as protection against herbivores by plants in the family Brassicaceae, while in vertebrates, ingested glucosinolates exert health-promoting effects due to their antioxidant and detoxifying properties as well as effects on cell proliferation and growth. The aim of this study was to investigate if Atlantic salmon fed two different doses of glucosinolate-enriched feeds would be protected against lice infection. The effects of feeding high dose of glucosinolates before the infection, and of high and low doses five weeks into the infection were studied.

Methods: Skin was screened by 15 k oligonucleotide microarray and qPCR.

Results: A 25 % reduction ($P < 0.05$) in lice counts was obtained in the low dose group and a 17 % reduction in the high dose group compared to fish fed control feed. Microarray analysis revealed induction of over 50 interferon (IFN)-related genes prior to lice infection. Genes upregulated five weeks into the infection in glucosinolate-enriched dietary groups included Type 1 pro-inflammatory factors, antimicrobial and acute phase proteins, extracellular matrix remodeling proteases and iron homeostasis regulators. In contrast, genes involved in muscle contraction, lipid and glucose metabolism were found more highly expressed in the skin of infected control fish.

Conclusions: Atlantic salmon fed glucosinolates had a significantly lower number of sea lice at the end of the experimental challenge. Feeding glucosinolates coincided with increased expression of IFN-related genes, and higher expression profiles of Type 1 immune genes late into the infection. In addition, regulation of genes involved in the metabolism of iron, lipid and sugar suggested an interplay between metabolism of nutrients and mechanisms of resistance.

Keywords: Atlantic salmon, *Salmo salar*, Sea lice, Anti-attachment feed, Glucosinolates

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Background

Sea lice infections constitute a major and global problem for salmonid aquaculture. Infection control relies primarily on chemical treatments whose repertoire is limited because of resistance to existing anti-parasitic compounds [1]. In addition, stress to the fish caused by frequent delousing events is of particular concern [2]. In order to apply the optimal treatment, resistance monitoring in lice populations, implemented through a nationwide surveillance program in Norway, could be helpful [1–4]. The amount of chemotherapeutants used against lice in Norway has surged over recent years; in 2008, 218 kg (kg of active substance, excluding hydrogen peroxide) were used, compared to 6,810 kg in 2012, 8,403 kg in 2013 and 12,812 kg in 2014 [5]. Despite differences in the dosage of used chemotherapeutants and increase in the general production of Atlantic salmon, the rise in the number of treated salmon is likely the result of resistance development in sea lice [3, 6]. Finding alternative strategies for managing lice infections is therefore becoming increasingly more important.

Salmon lice (*Lepeophtheirus salmonis*) immunomodulate their hosts by secreting a complex cocktail of bioactive compounds [7–9]. Release of these secretory/excretory products depends on the host species, being highest in response to Atlantic salmon [10]. This is in line with comparative studies showing that Atlantic salmon is among the most susceptible salmonid species [11–16]. Activation of the Type 1 immunity, similar to mammalian Type 1 (Th1 and Th17 responses), could play a role in the resistance of salmonids to lice infections [12, 13, 17, 18], especially during early stages of infection [12]. Pro-inflammatory Type 1 responses (and to a lesser extent Type 2 responses) in skin were negatively correlated to the number of *L. salmonis* chalimus stages in Atlantic salmon [17]. Skewing of immunity towards the Type 2 immunophenotype with a strong immunosuppressive component in Atlantic salmon (termed Th2-modified) likely contributes to susceptibility of Atlantic salmon [18], while Type 2 responses seem to have a more beneficial role in coho salmon during later stages of infection with *L. salmonis* [12].

There are several encouraging examples of the use of orally delivered microbial immunostimulants that promote protective immune responses [19–22]. The use of dietary plant-derived bioactives is also considered a promising approach. Plants in the family Brassicaceae contain secondary metabolites called glucosinolates (GLs) that protect against herbivory [23], bacterial and fungal disease agents [24–26]. When plant cells are destroyed by chewing or other mechanical processing, the enzyme myrosinase comes into contact with GLs and hydrolyses them into isothiocyanates (ITCs). These compounds act as insect deterrents but might also be toxic to invertebrates upon ingestion [25, 27]. Their strong pungent flavor [28] may also mask the host smell and obscure the host recognition and/or attachment process by sea lice. A range of olfactory receptors have been identified in both *L. salmonis* and *Caligus rogercresseyi* [29–31].

Studies in mammals have revealed that GLs-derived ITCs exert chemopreventive effects mainly attributed to induction of antioxidant and detoxification pathways (reviewed in [32, 33]). A majority of in vivo and in vitro studies report anti-inflammatory effects of ITCs in a range of pathological conditions, organs and cell lines, including tumor cells [32, 34]. However, pro-inflammatory type 1 responses have also been seen in murine skin after exposure to ITCs [35], suggesting an organ dependent regulation of immune responses by ITCs.

To date, GLs and their breakdown products have not been investigated as feed additives against aquatic parasites of Atlantic salmon. In this study, we hypothesised that dietary GLs would modulate skin immune and physiological responses, prior to and during lice infection, thus interfering with the attachment and establishment of *L. salmonis*.

Methods

Ethics statement

The experimental facilities used in this study at Ewos Innovation, Dirdal, Norway, number 131 was approved by the Norwegian Animal Research Authority 02.02.2012 until 25.01.16. The experiments/procedures have been conducted in accordance with the laws and regulations controlling experiments/procedures in live animals in Norway, e.g. the Animal Welfare Act of 20th December 1974, No 73, chapter VI sections 20–22 and the Regulation on Animal Experimentation of 15th January 1996.

Fish trials, production of feeds and copepodids

All trials were performed at Ewos Innovation's Test Facility in Dirdal, Norway from October to December 2012. Fish tanks used in all trials were 500 l circular flow-through tanks with an average temperature and salinity of 8.7 °C and 27.4 ppt, respectively.

The feeds used in this study were produced at the Ewos Innovation plant in Dirdal, Norway. The fish were fed with the control feed (C) OPAL (EWOS Opal, EWOS, Norway) and two experimental (anti-attachment) feeds that contained GLs. The low dose feed (LD) had 3.61 % and the high dose feed (HD) had 13.0 % of the GLs-containing ingredient originating from a plant of the family Brassicaceae, with the approximate GLs content of 7.3 μmol/g and 26.4 μmol/g in LD and HD, respectively (see Additional file 1: Table S2 for details of the dietary composition). All diets had a pellet size of 5.5 mm.

To examine the feed intake for the three diets, 30 fish in three tank replicates (90 per diet) were fed to satiation for three weeks (Fig. 1a). The amount of uneaten pellets was measured weekly. After the feed intake study, all tanks were fed OPAL and the numbers of fish in each tank was reduced from 30 to 20 (acclimation period). After 10 days of control feeding, six of the tanks were fed the anti-attachment LD and HD feeds and three tanks continued on C for 12 days (pre-infection period), and throughout the 31–35 days of *L. salmonis* infection (post-infection period). The treatment groups tested in this part of the study were named: infected C (I-C) infected LD (I-LD) and infected HD (I-HD) in three tank replicates. After feeding the experimental diets for 12 days (pre-infection period), fish were infected with 50 copepodids per fish by turning off the water flow and lowering water level to 15 cm height before copepodids were evenly distributed to the nine fish tanks. Oxygen was added using a fine ceramic diffusor, with individual air valves controlling the oxygen flow to each tank. After 1 h of exposure, water flow was resumed. Lice counting and sampling were done when majority of lice reached preadult stages. During a sampling period of four days, number, stage and gender of lice on each fish were recorded. In addition, skin samples from 3 fish from each tank (9 fish from each group), approximately 5 × 5 mm in size, were excised from the site immediately caudally of the dorsal fin, and put in RNA*later* (Ambion®, Austin, TX, USA) at 4 °C for 24 h and then stored at -80 °C until further processing. Fish weights and lengths, and the presence of feces were also registered. Lice counts were analysed by one-way ANOVA with Tukey's *post-hoc* test using the GraphPad Prism 6.0 software. Fish performance and distribution of life stages were analysed by using Microsoft Excel 2010. Fulton's condition factor was calculated by the formula: (100 $BWFL^{-3}$) [36].

Six tanks of fish were used in a parallel feed study (Fig. 1b) to assess the effect of GLs feeding *per se* (without infection). After one month of feeding control feed

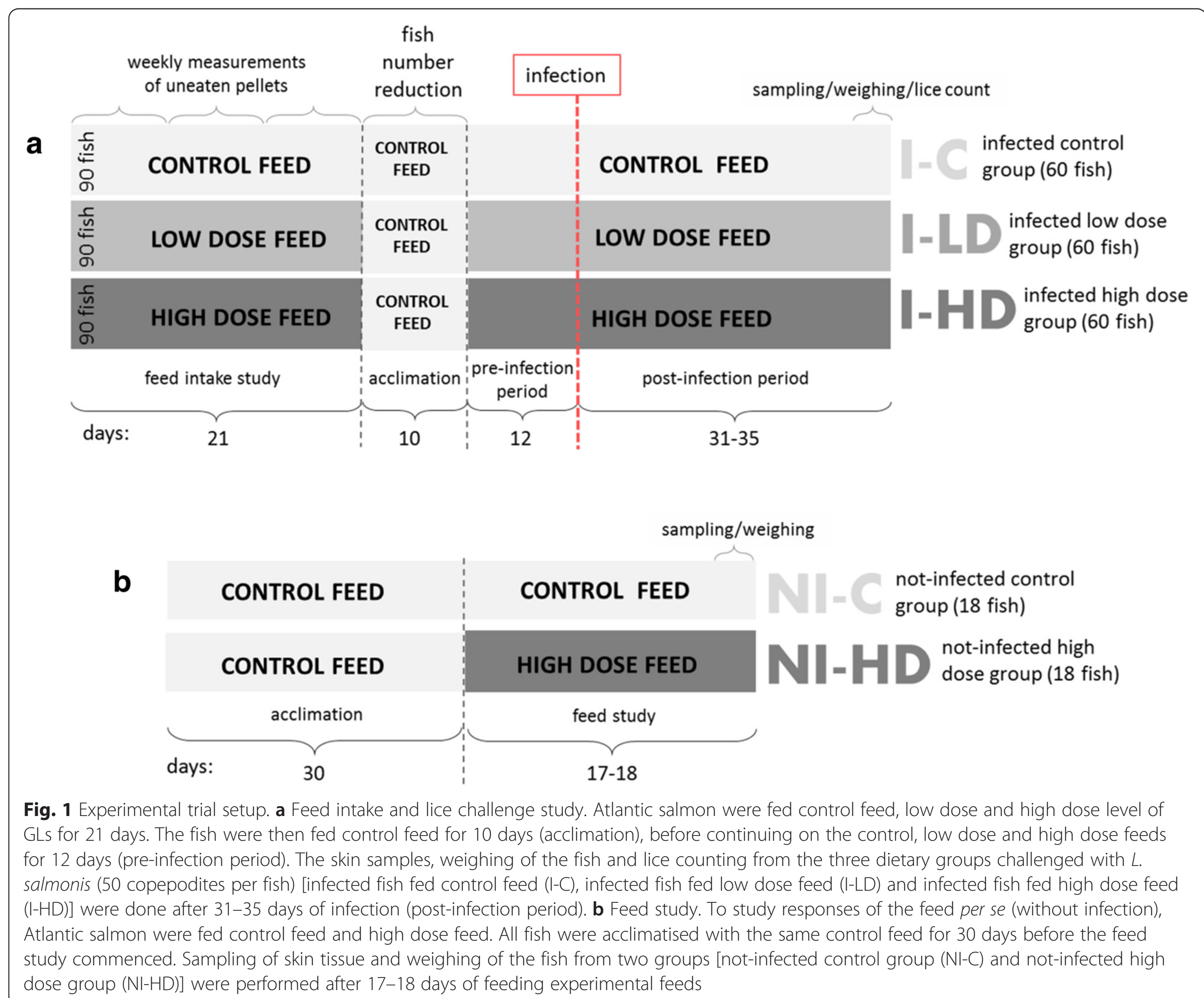


Fig. 1 Experimental trial setup. **a** Feed intake and lice challenge study. Atlantic salmon were fed control feed, low dose and high dose level of GLs for 21 days. The fish were then fed control feed for 10 days (acclimation), before continuing on the control, low dose and high dose feeds for 12 days (pre-infection period). The skin samples, weighing of the fish and lice counting from the three dietary groups challenged with *L. salmonis* (50 copepodites per fish) [infected fish fed control feed (I-C), infected fish fed low dose feed (I-LD) and infected fish fed high dose feed (I-HD)] were done after 31–35 days of infection (post-infection period). **b** Feed study. To study responses of the feed *per se* (without infection), Atlantic salmon were fed control feed and high dose feed. All fish were acclimatised with the same control feed for 30 days before the feed study commenced. Sampling of skin tissue and weighing of the fish from two groups [not-infected control group (NI-C) and not-infected high dose group (NI-HD)] were performed after 17–18 days of feeding experimental feeds

(acclimation), three tanks of fish were fed high dose (HD) diet. The other three tanks continued on control feed. These groups of fish were named not-infected high dose (NI-HD) and not-infected control (NI-C), respectively. Sampling of skin tissue was performed after 17–18 days of feeding using the same protocol as for the infected fish; skin tissue of 9 fish from each group were sampled, and weights and length of 18 fish from each group were registered.

Salmon lice (*L. salmonis*) used in this trial were collected from Oltesvik (Norway) in March 2012. To provide a predictable supply of lice for future trials, this lice population was propagated and maintained on Atlantic salmon hosts kept in the *L. salmonis* cultivation system in the Sea Lice lab at the Dirdal facility, which provided stable supply of robust wild-type lice. Lice and host fish were held in 850 l circular flow-through tanks and egg strings from egg-bearing females were collected from anaesthetised salmon. The anesthetic used was Finquel (100 mg/l, Scan Aqua, Årnes, Norway). During an incubation period of 14 days (9 °C), the egg strings were allowed to hatch and reach the infective copepodid stage. The number of copepodids was counted in a zooplankton-counting chamber to calculate the density. At least four samples of 50 ml each were taken to improve the accuracy of estimation.

RNA extraction and cDNA synthesis

Total RNA extraction was done using the RNAeasy Mini Kit (Qiagen, Hilden, Germany) after adding Trizol (GIBCO, Life Technologies, Carlsbad, CA, USA) and homogenizing 50 mg of samples with 1.4 mm zirconium oxide beads (VWR, Oslo, Norway). After this, chloroform was added, samples were centrifuged and the RNA supernatant was subsequently subjected to RNA cleanup according to Qiagen protocol. The concentration of RNA was determined by spectrophotometry using NanoDrop ND1000 (Nanodrop Technologies, Wilmington, DE, USA) and stored at -80 °C until further use. The integrity of total RNA was determined using an Agilent 2100 Bioanalyzer, and only samples with RNA integrity number (RIN) of 8 or higher were accepted. Genomic DNA contamination was excluded by performing qPCR reactions using isolated RNA as templates together with primers for elongation factor-1α (*EF1A*).

Microarray hybridization and data processing

Five fish from each group (I-C, I-LD, I-HD and NI-C), and four fish from NI-HD were analysed by 15 k Atlantic salmon microarray (SIQ6); these individuals were a subset of fish analysed by qPCR. All samples were compared to pooled reference RNA that consisted of two fish from all groups, except for I-LD. The test samples labelled with Cy5 and pooled reference with Cy3 were competitively hybridised to array slides. All reagents and equipment used for microarray analyses were from Agilent Technologies; protocols were used according to the manufacturer. Labelling and amplification of RNA was performed on 100 ng total RNA using Two-colour Quick Amp Labelling kits. Gene Expression Hybridization Kit was used for the fragmentation. Hybridizations were performed in a rotation oven for 17 h at 65 °C with rotation speed of 10 rpm, followed by 1 min washing of arrays with Gene Expression Wash Buffer I at room temperature and Gene Expression Wash Buffer II at 37 °C. To achieve an overall intensity ratio close to 1 between Cy3 and Cy5 channels with minimal saturation, the slides were scanned immediately using GenePix Personal 4100A scanner (Molecular Devices, Sunnyvale, CA, USA) at 5 μm resolution and with manually adjusted laser power. For feature extraction of fluorescence intensity values and assessment of spot quality that followed spot-grid alignment, the GenePix pro software 6.0 was used. Subsequent to filtration of low quality spots flagged by the software, Lowess normalization of $\log_2$-expression ratios (ER) was performed. Differentially expressed genes (DEGs) were selected by comparison with the not-infected control (NI-C): $\log_2$-ER > 0.6 and $P < 0.05$ in at least one group were the criteria used. Fold values of $\log_2$-ERs (DEGs) were then calculated. Nofima's bioinformatics system (STARS) was used for data analyses.

qPCR protocol

To validate the microarray data and screen other genes of interest (see Additional file 1: Table S1 for the full list), 9 fish from each group (I-C, I-LD, I-HD and NI-HD), in addition to NI-C group of fish were analysed by qPCR. For each sample, 1,800 ng of RNA was used to synthesize cDNA using the cDNA Affinity Script (Agilent Technologies, Matriks AS, Oslo, Norway) following the manufacturer's protocol. Every reaction contained 1 μl of random primers and 2 μl of oligo DT primers. Each gene was run in duplicates by adding 4 μl of 1:10 diluted cDNA from each fish, 1 ul of each primer (10 μM concentration) (see sequences in Additional file 1: Table S1) and LightCycler 480 SYBR Green I Master mix (Roche) to a final volume of 12 μl in 96-well plates. Cycling conditions in LightCycler 480 instrument (Roche, Applied Science) were 5 min denaturation step at 95 °C, 40 cycles of denaturation (10 s at 95 °C), annealing (20 s at 60 °C) and extension (15 s at 72 °C), followed by melting curve analysis with measurements of the fluorescence performed in the temperature range between 65–97 °C. The maximum-second-derivative method (Roche diagnostics) was used to find the crossing point (Cp) value. The relative expression of target genes was calculated by using the ΔΔCt method. The reference gene *EF1A* was selected as it is one of the

most well-established reference gene in studies of Atlantic salmon tissues in general [37] as well as in lice infected tissues [12, 17, 21, 38–40]. In this study, the mean Cp value in each group varied less than 0.5 cycle. One-way ANOVA with subsequent Tukey's multiple comparisons test in the GraphPad Prism Software were executed between each of the 5 groups. Specificity and efficiency were confirmed by melting curve analysis and two-fold serial dilutions of cDNA for each primer pair in triplicates, respectively. PCR efficiency for all genes ranged from 1.8–2.

Results

Fish performance and lice counts

No changes in appetite were observed for any of the diet groups during the study period. Only two fish from the control group died during the trial period (of non-specific causes). The fish weights [mean (g) ± SD] at the end of the lice-challenge (Fig. 1a) were as follows: I-C: 871 ± 127; I-LD: 751 ± 121; and I-HD: 726 ± 113, where the two latter groups differed significantly from the control group (I-LD *vs* I-C: *t*-test: $t_{(38)} = 3.62$, $P = 0.0009$; I-HD *vs* I-C: *t*-test: $t_{(38)} = 4.96$, $P < 0.0001$). However, the condition factor (mean ± SD) was lower in control than in fish exposed to GLs: I-C group (1.43 ± 0.13), I-LD (1.54 ± 0.16) and I-HD (1.52 ± 0.13) (I-LD *vs* I-C: *t*-test: $t_{(38)} = 2.255$, $P = 0.030$; I-HD *vs* I-C: *t*-test: $t_{(38)} = 1.65$, $P = 0.10$). In the feed study (Fig. 1b), neither weights nor condition factors differed significantly between the NI-C group and NI-HD (weight *t*-test: $t_{(34)} = 0.49$, $P = 0.62$; condition factor *t*-test: $t_{(34)} = 1.37$, $P = 0.18$). Lice counts and tissue sampling were performed at five weeks post-infection, when most of the female lice had reached pre-adult 2 stage. The distribution of different life stages was not affected by the diet (Table 1).

Lice were counted on 180 fish (60 fish per dietary group), and the average number of lice ± SD was 15.4 ± 5.3 (range 4–35). The mean number of lice in each group was I-C: 18 ± 5.1; I-LD: 13.5 ± 4.8; and I-HD: 15 ± 4.6. Compared to infected control fish (I-C), there was a 25 % reduction in lice number in the I-LD group, while I-HD had 17 % less lice (Table 2). One-way ANOVA with Tukey's *post-hoc* test (ANOVA: $F_{(2, 177)} = 14.39$, $P < 0.0001$) showed significant difference in lice number between I-LD and I-C ($P < 0.0001$), as well as between I-HD and I-C ($P = 0.0036$).

Gene expression responses to GLs, lice and their combinations: microarray analyses

In GLs-containing feed groups, lice infection resulted in the increased expression of multiple genes and a large part of the upregulated genes was categorised as immune genes. A similar trend of expression among genes that were grouped according to their biological function was found within, but not between the treatments (Figs. 2 and 3). The largest group (66 features, Fig. 2a-c) consisted of genes associated with innate antiviral immunity [41]. In this study, innate antiviral genes were strongly upregulated in the not-infected GLs-group (NI-HD) compared to NI-C, lice infection resulted in slight downregulation in I-C, while I-LD and I-HD showed intermediate values (Fig. 2c). One example is myxovirus resistance 1 that was upregulated 2.86-fold in NI-HD, downregulated following lice infection in I-C, while being significantly increased (1.61-fold) in I-HD (Fig. 2c). Receptor transporting protein 3 was on the top of the list with 4.6-fold upregulation in NI-HD compared to NI-C (Fig. 2c). To note was also the concerted induction of several GTPases and GTP binding proteins, which are known as important components of the cellular antiviral response. Slight GLs-mediated induction of genes involved in antigen presentation was suppressed after lice infection, with no recovery five weeks into the infection when most lice reached the preadult stage (Fig. 2d).

Activation of the acute phase response genes in skin included increased expression in NI-HD and I-HD of serum amyloid A, A5 and amyloid beta A4 compared to NI-C (Fig. 2d). On the other side, lowest expression of genes encoding proteins with diverse transport and scavenger functions (many of which are classified as negative acute phase plasma proteins) was found in I-LD (Fig. 2d). Most other immune genes were also induced, either by GLs, lice or both, when compared to NI-C (Table 2).

Majority of genes affected by GLs in infected groups showed dose-dependent responses: changes in I-HD were either equal to or greater than I-LD. However, several matrix metalloproteinase (*MMP*) genes, critical for extracellular remodeling during wound healing and inflammation [42] reached maximum expression levels in I-LD. Increased *MMP13* expression has been linked to

Table 1 Distribution of gender and life stages of lice found on Atlantic salmon in the infected control group (I-C), infected group fed low inclusion level of GLs (I-LD) and infected group fed high inclusion level of GLs (I-HD)

Dietary group/stage	Preadult 1 (males)	Preadult 1 (females)	Preadult 2 (males)	Preadult 2 (females)	Adult (males)
I-C	0.55	2.02	8.46	46.18	42.78
I-LD	0.37	2.48	7.43	46.53	43.19
I-HD	0.11	1.33	8.23	46.83	43.38

Variables are shown as percentages of the total (100 %) lice count for each group

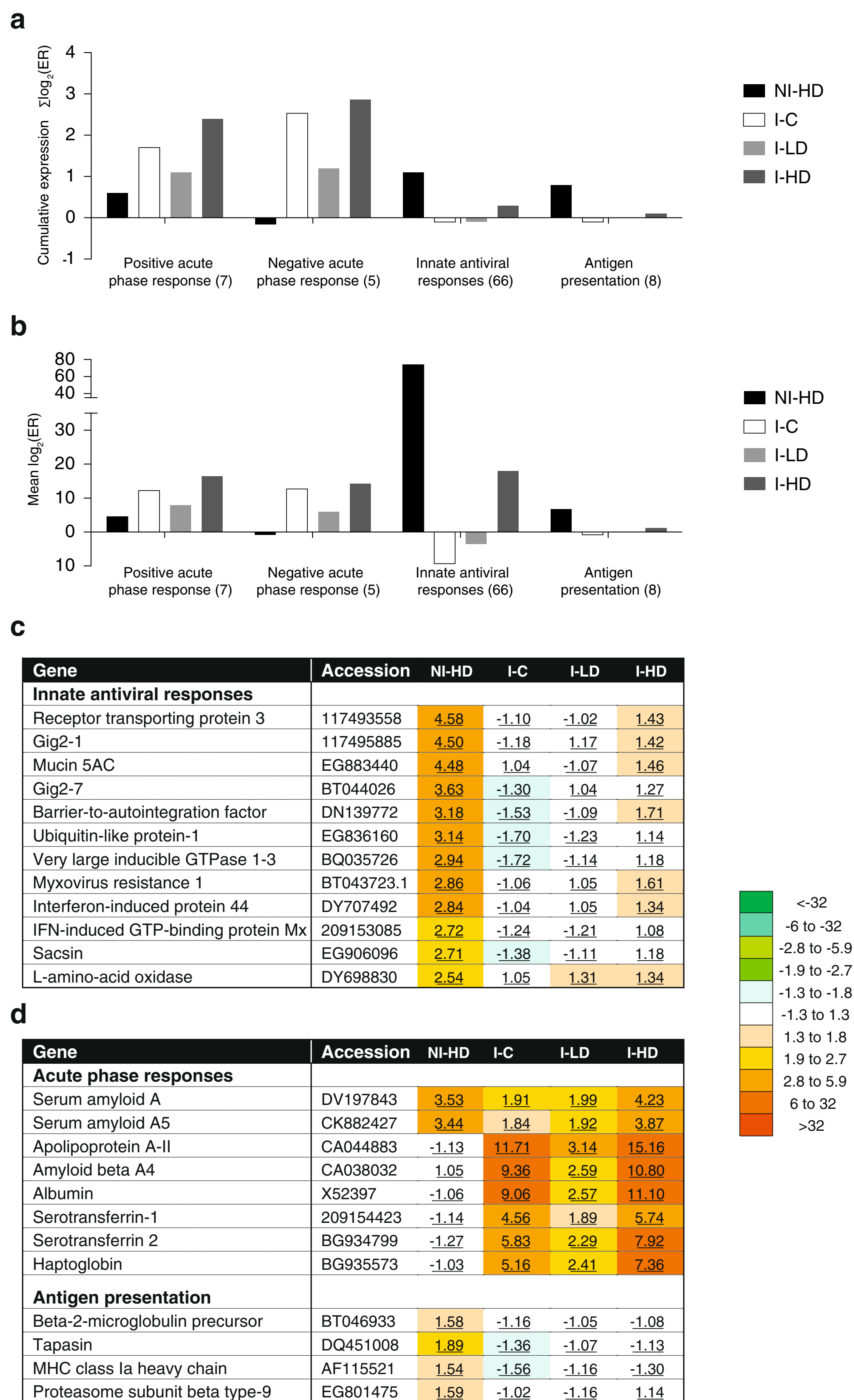
Table 2 Examples of immune genes with differential expression in skin (microarray results)

Gene	Abbreviation	Accession	NI-HD	I-C	I-LD	I-HD
Chemokines, cytokines and receptors						
C-C motif chemokine 19-1	*CCL-C5A*	209737465	2.87	1.17	1.53	2.03
C-C motif chemokine 19-2	*CCL-C5A*	117433169	2.87	1.15	1.49	1.95
C-C motif chemokine 13		EG872936	2.33	1.39	1.41	1.87
CXCL10-like chemokine		EF619047	3.73	-1.40	-1.04	1.35
Leukocyte cell-derived chemotaxin 2-1	*BX005069.2*	117545301	1.04	5.76	2.47	7.35
Small inducible cytokine A13	*CCL13*	GE835061	2.67	1.02	1.48	2.65
C-C chemokine receptor type 3	*CCR12.3*	223648789	2.11	1.20	1.47	1.97
Interleukin-20 receptor alpha chain	*CRFB2*	117446659	4.94	1.07	1.17	1.38
Effectors						
TNFa induced metalloreductase STEAP4		223649457	1.58	-1.19	2.12	1.53
Neutrophil cytosolic factor 1	*NCF1*	223647567	1.31	1.32	2.17	2.03
RNase 1	*RNASE1*	DN047839	-1.10	8.63	2.74	11.34
Granzyme A	*GZMA*	209733889	2.95	-1.01	1.01	1.74
Complement factor H1 protein	*CFH1*	DY713380	1.01	6.02	1.95	7.39
Antimicrobial peptide NK-lysin		EG840346	2.93	1.61	2.41	1.68
Natterin-like protein	*NATTL*	223584499	-1.01	1.08	2.29	2.50
C1q-like specific protein	*CBLN8*	117537786	9.46	21.86	31.38	106.26
Cathelicidin antimicrobial peptide 2	*CATH-2*	AY360357	1.87	2.20	3.62	3.46
Collagenase 3	*MMP13A*	AJ424540	3.21	3.66	7.63	5.22
MMP 13 or Collagenase 3	*MMP13A*	209156091	2.97	2.67	7.34	4.57
Lectins and coreceptors						
Mannose-specific lectin		209733483	2.68	3.31	6.34	9.57
P-selectin	*SELE*	BT058751	2.53	1.28	1.45	1.81
C type lectin receptor A		AY572832	2.17	2.55	3.18	2.74
Leukolectin protein		60377755	1.26	2.51	1.35	-1.11
CD83	*CD83*	DQ339141	2.13	1.01	-1.28	1.25
CD97 antigen	*CABZ01066772*	EG935955	1.81	1.22	1.55	1.92
CD209 antigen-like protein E		S35562993	2.17	-1.07	-1.03	1.00

Data are mean fold calculated from log_2 (ER) values and compared to NI-C. Values with significant difference compared to the NI-C group are underlined

lice resistance in our previous study [17]. In this study, C1q-like specific protein showed the overall greatest expression change (31.38-fold) in the best-protected I-LD group compared to NI-C. Natterin-like protein (*NATTL*) is a homologue of *NATTL* gene in *T. nattereri* that codes for a protein found in the venomous secretions of this fish species [43] and neutrophil cytosolic factor 1 were stimulated only by combinations of GLs and lice but at much lower levels. The upregulated immune genes included a number of inflammatory mediators. Two C-C motif chemokines 19 (*CCL19*), which responded to GLs might have a role in T cell proliferation and maturation of DCs that promote Th1 rather than Th2 responses [44]. Similar profile was seen for several other chemokines and receptors (Table 2). A Th2 marker C-C chemokine receptor type 3 [45] was more highly expressed in GLs groups, a similar trend was shown by granzyme A, an effector molecule of T cells. Furthermore, the small inducible cytokine A13 (*CCL13*) (2.65-fold induced) is a chemoattractant for a diverse group of immune cells [46]. High (5-fold) induction of IL-20 receptor alpha chain precursor (4.95-fold) in NI-HD, followed by 1.38 fold in I-HD was noteworthy as this receptor transduces highly pro-inflammatory signals in mammalian skin [47]. The leukocyte cell-derived chemotaxin 2 was upregulated by lice (I-C) and even more so in I-HD, and is associated with responses to lice [18] and lice resistance [17] in our previous studies.

Genes for diverse innate effectors were also activated. Golgi-residing metalloreductase STEAP family member 4, associated with iron metabolism and inflammation [48, 49] responded only to GLs, being most highly induced in I-LD,



c

Gene	Accession	NI-HD	I-C	I-LD	I-HD
Innate antiviral responses					
Receptor transporting protein 3	117493558	4.58	-1.10	-1.02	1.43
Gig2-1	117495885	4.50	-1.18	1.17	1.42
Mucin 5AC	EG883440	4.48	1.04	-1.07	1.46
Gig2-7	BT044026	3.63	-1.30	1.04	1.27
Barrier-to-autointegration factor	DN139772	3.18	-1.53	-1.09	1.71
Ubiquitin-like protein-1	EG836160	3.14	-1.70	-1.23	1.14
Very large inducible GTPase 1-3	BQ035726	2.94	-1.72	-1.14	1.18
Myxovirus resistance 1	BT043723.1	2.86	-1.06	1.05	1.61
Interferon-induced protein 44	DY707492	2.84	-1.04	1.05	1.34
IFN-induced GTP-binding protein Mx	209153085	2.72	-1.24	-1.21	1.08
Sacsin	EG906096	2.71	-1.38	-1.11	1.18
L-amino-acid oxidase	DY698830	2.54	1.05	1.31	1.34

d

Gene	Accession	NI-HD	I-C	I-LD	I-HD
Acute phase responses					
Serum amyloid A	DV197843	3.53	1.91	1.99	4.23
Serum amyloid A5	CK882427	3.44	1.84	1.92	3.87
Apolipoprotein A-II	CA044883	-1.13	11.71	3.14	15.16
Amyloid beta A4	CA038032	1.05	9.36	2.59	10.80
Albumin	X52397	-1.06	9.06	2.57	11.10
Serotransferrin-1	209154423	-1.14	4.56	1.89	5.74
Serotransferrin 2	BG934799	-1.27	5.83	2.29	7.92
Haptoglobin	BG935573	-1.03	5.16	2.41	7.36
Antigen presentation					
Beta-2-microglobulin precursor	BT046933	1.58	-1.16	-1.05	-1.08
Tapasin	DQ451008	1.89	-1.36	-1.07	-1.13
MHC class Ia heavy chain	AF115521	1.54	-1.56	-1.16	-1.30
Proteasome subunit beta type-9	EG801475	1.59	-1.02	-1.16	1.14

Fig. 2 (See legend on next page.)

(See figure on previous page.)

Fig. 2 Immune genes with correlated expression profiles (microarray results). **a** Cumulative expression changes assessed as $\Sigma \log_2$ (ER). **b** Mean $\log_2$ (ER); numbers of features are in parentheses. **c** Tabulated examples of most regulated genes involved in innate antiviral responses. Data are mean fold calculated from $\log_2$ (ER) values and compared to NI-C (significantly different values are underlined. **d** Tabulated examples of the most regulated genes involved in positive and negative acute phase response and antigen presentation. Data are mean fold calculated from $\log_2$ (ER) values and compared to NI-C (significantly different values are underlined)

suggesting links between iron regulation, inflammation and resistance to lice. The antimicrobial peptides NK-lysin and cathelicidin antimicrobial peptide 2 that have been correlated to lice resistance in our previous studies [17, 18, 39] were stimulated by both diet and lice infection, and their combination, while RNase 1, a cell-cidal effector and complement regulatory factor H1 were upregulated only by lice infection (I-C). Several lectins and lectin co-receptors present on leucocytes were induced by GLs (p-selectin, *CD83*, *CD209* and *CD97*) or by a combination of lice and GLs (mannose-specific lectin and C type lectin receptor A).

Apoptotic and stress responses to GLs and lice in skin of Atlantic salmon were relatively weak (Table 3). Caspase-3 showed slight induction, while synergistic downregulation was observed for pro-apoptotic ` switch protein 2 [50]: 4-fold in I-LD compared to NI-C and 4.3-fold in I-HD. Upregulation by both lice and GLs was seen in two genes encoding heat-shock proteins and glutathione peroxidase, a scavenger of free radicals. Hydrogen peroxide producing enzyme *L*-amino-acid oxidase was induced only by GLs.

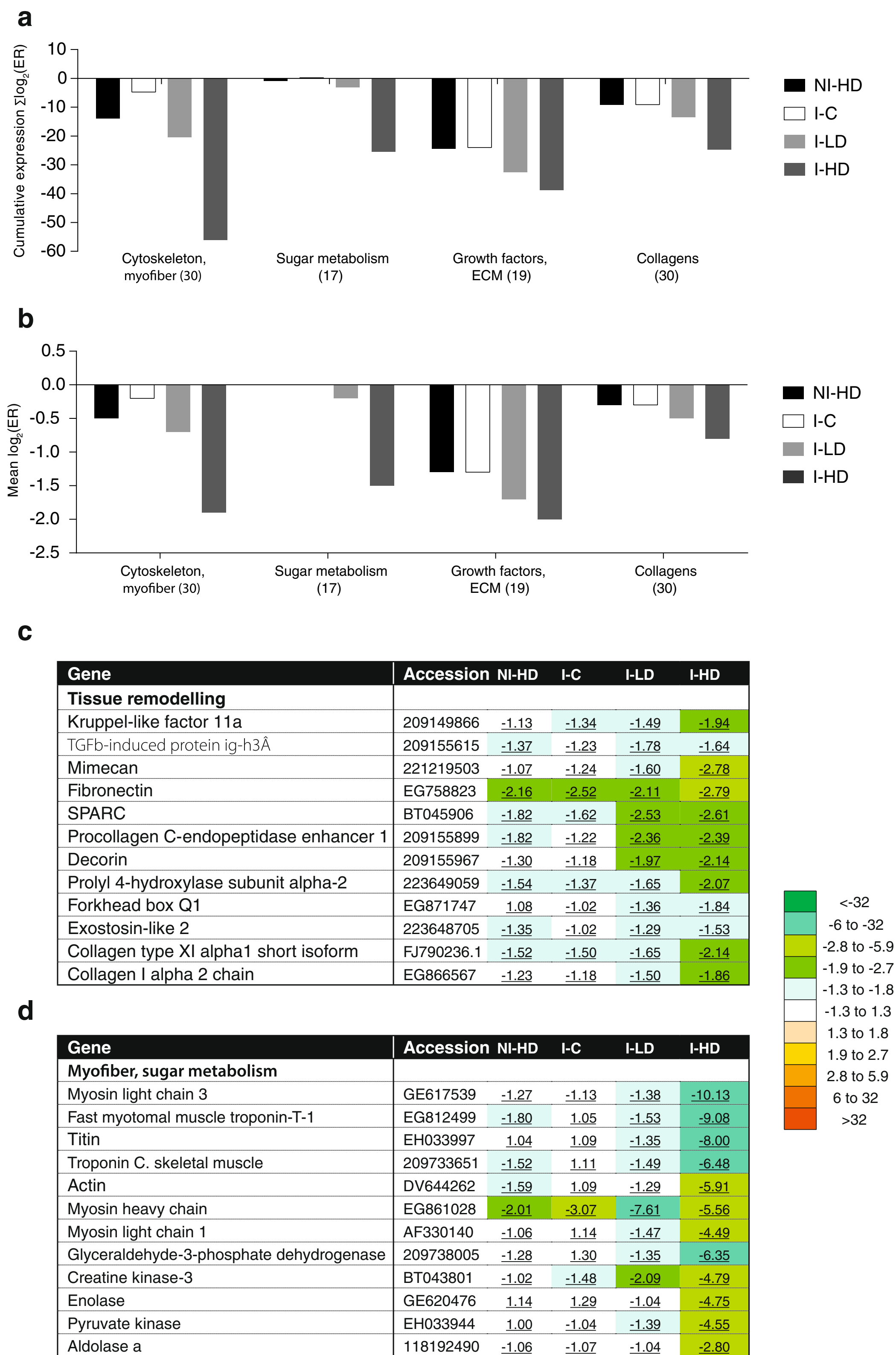
Differential expression was seen in multiple genes encoding intracellular fibrous structural proteins. Joint treatments (diet and lice infection) induced several genes involved in keratinization (Table 3). Keratin type I cytoskeletal 17 and type II keratin E3 are parts of the epithelial cytoskeleton, which provides mechanical resilience of epithelial cells and in addition can be involved in intracellular signaling [51]. Many more genes were downregulated and several functional groups showed highly coordinated expression changes (Fig. 3a, b). Two clusters of co-expressed genes included myofiber proteins and enzymes of sugar metabolism (30 and 17 features, respectively); higher concentration of GLs produced stronger downregulation in both groups. Myofiber genes included mainly components of the myocontractile apparatus: myosin light and heavy chains, actin, troponins and titin (Fig. 3a, b, d). Similar though weaker changes were observed in lipid and steroid metabolism (Table 3). A number of genes with roles in tissue differentiation, formation of extracellular matrix (ECM) and wound healing, including multiple collagens, transcription factors forkhead box Q1 and kruppel-like factor 11a, receptor exostosin-like 2 [52, 53] and transforming growth factor-beta-induced protein ig-h3 [54] were downregulated by both GLs and lice infection (Fig. 3c). The effect was slightly enhanced by the combination of lice and high dose of GLs.

qPCR results

Real time qPCR results are shown in Fig. 4. Expression of genes of interest are shown in Fig. 4b, c and the differential expression of *BANF* (ANOVA: $F_{(4,40)} = 7.350$, $P = 0.0002$), *CXCL10* (ANOVA: $F_{(4,40)} = 3.147$, $P = 0.02$ 43), *LECT2* (ANOVA: $F_{(4,40)} = 7.171$, $P = 0.0002$), *ZG16* (ANOVA: $F_{(4,40)} = 2{,}214$, $P = 0.0848$) and cathelicidin (ANOVA: $F_{(4,40)} = 5.421$, $P = 0.0014$) measured by the array, were validated by qPCR (Fig. 4a). Both diet and lice infection modulated skin transcriptional responses related to immunity. For most pro-inflammatory genes expression was lowest in I-C (Fig. 4b). qPCR analysis confirmed that fish groups exposed to GLs-diets had a significantly higher increase in interferons namely *IFNy*, compared to the NI-C group (ANOVA: $F_{(4,40)} = 4.377$, $P = 0.0050$; NI-HD *vs* NI-C: $P = 0.04$; I-LD *vs* NI-C: $P = 0.01$; I-HD *vs* NI-C: $P = 0.0070$). The almost double increase in the I-HD group of complement component *C3* [55] (Fig. 4c) (ANOVA: $F_{(4,40)} = 9.761$, $P < 0.0001$; I-C *vs* I-HD: $P = 0.76$) and neutrophil attractant *IL8* [56] (Fig. 4b) (ANOVA: $F_{(4,40)} = 19.24$, $P < 0.0001$; I-C *vs* I-HD: $P = 0.145$), compared to I-C group was also observed. Expression of the neutrophil marker myeloperoxidase (*MPO*) [57] (Fig. 4c) (ANOVA: $F_{(4,40)} = 5.3$, $P = 0.0016$) and neutrophil chemoattractant *IL17A* [58, 59] (Fig. 4b) (ANOVA: $F_{(4,40)} = 3.088$, $P = 0.026$) was remarkable; they were both suppressed in NI-HD but most highly induced upon infection in fish exposed to GLs-enriched feeds. Interestingly, *IL4/13*, a putative Th2 cytokine in fish [60] (Fig. 4b) was found to be most highly induced in I-HD (ANOVA: $F_{(4,40)} = 19.66$, $P < 0.0001$; I-HD *vs* NI-C: $P < 0.0001$), likely suggesting the need to counteract Type 1 immunity and fine tune highly pro-inflammatory immune responses.

Discussion

The use of anti-attachment feeds promises to be a safe, easy to administer and cost-effective approach against sea lice. The achieved reduction in parasite numbers amounted to 25 % and could thus only be complementary to other control measures within the integrated pest management. Further work is needed to determine the optimal dosage and other possible effects that the



c

Gene	Accession	NI-HD	I-C	I-LD	I-HD
Tissue remodelling					
Kruppel-like factor 11a	209149866	-1.13	-1.34	-1.49	-1.94
TGFb-induced protein ig-h3Â	209155615	-1.37	-1.23	-1.78	-1.64
Mimecan	221219503	-1.07	-1.24	-1.60	-2.78
Fibronectin	EG758823	-2.16	-2.52	-2.11	-2.79
SPARC	BT045906	-1.82	-1.62	-2.53	-2.61
Procollagen C-endopeptidase enhancer 1	209155899	-1.82	-1.22	-2.36	-2.39
Decorin	209155967	-1.30	-1.18	-1.97	-2.14
Prolyl 4-hydroxylase subunit alpha-2	223649059	-1.54	-1.37	-1.65	-2.07
Forkhead box Q1	EG871747	1.08	-1.02	-1.36	-1.84
Exostosin-like 2	223648705	-1.35	-1.02	-1.29	-1.53
Collagen type XI alpha1 short isoform	FJ790236.1	-1.52	-1.50	-1.65	-2.14
Collagen I alpha 2 chain	EG866567	-1.23	-1.18	-1.50	-1.86

d

Gene	Accession	NI-HD	I-C	I-LD	I-HD
Myofiber, sugar metabolism					
Myosin light chain 3	GE617539	-1.27	-1.13	-1.38	-10.13
Fast myotomal muscle troponin-T-1	EG812499	-1.80	1.05	-1.53	-9.08
Titin	EH033997	1.04	1.09	-1.35	-8.00
Troponin C. skeletal muscle	209733651	-1.52	1.11	-1.49	-6.48
Actin	DV644262	-1.59	1.09	-1.29	-5.91
Myosin heavy chain	EG861028	-2.01	-3.07	-7.61	-5.56
Myosin light chain 1	AF330140	-1.06	1.14	-1.47	-4.49
Glyceraldehyde-3-phosphate dehydrogenase	209738005	-1.28	1.30	-1.35	-6.35
Creatine kinase-3	BT043801	-1.02	-1.48	-2.09	-4.79
Enolase	GE620476	1.14	1.29	-1.04	-4.75
Pyruvate kinase	EH033944	1.00	-1.04	-1.39	-4.55
Aldolase a	118192490	-1.06	-1.07	-1.04	-2.80

Fig. 3 (See legend on next page.)

(See figure on previous page.)
Fig. 3 Genes encoding cytoskeletal and myofiber (contractile) proteins, enzymes involved in sugar metabolism, growth factors and collagens with correlated expression profiles (microarray results). **a** cumulative expression changes assessed as $\Sigma \log_2$ (ER); **b** mean $\log_2$ (ER); numbers of features are in parentheses. **c** Tabulated are examples of the most regulated genes involved in myofiber and sugar metabolism. Data are mean fold calculated from $\log_2$ (ER) values and compared to NI-C (significantly different values are underlined). **d** Tabulated examples of most regulated genes involved in tissue remodeling. Data are mean fold calculated from $\log_2$ (ER) values and compared to NI-C (significantly different values are underlined)

bioactive compounds contained in anti-attachment feeds might exert on fish.

The goals of this study were to screen the transcriptomic response in skin after feeding Atlantic salmon diets enriched with GLs as well as to examine the impact of two inclusion doses of GLs (LD and HD) on the outcome of lice infection. One of the key findings in this study was the massive upregulation of a large group of genes involved in or associated with innate antiviral responses [41] in NI-HD (Fig. 2a-c) (the fish in this study showed no apparent signs of any viral disease). This is of note as the suppression of antiviral pathways by lice has been repeatedly reported [12, 16–19, 38, 40]. Innate anti-viral genes are co-regulated with interferons that play a key part in regulation of antiviral and antibacterial responses [61]. Stimulation of mice with ITCs resulted in increased expression level of canonical Th1 markers *IFN*γ and *T-bet* in the ear tissue [35]. Possible association between antiviral gene expression and reduced level of lice infection was shown in two of our previous studies. One addressed protection by sex steroid hormones, which conferred a 50 % reduction in lice counts [39] while in the other one, selective breeding based on 150 tested families resulted in a difference of around 36 % in lice counts between the top five extremely susceptible and resistant families included in the study [17]. The observations from the current study fit a previously suggested hypothesis, which states that responses similar to mammalian Type 1 pro-inflammatory responses (Th1/Th17) play a positive role in protection against *L. salmonis* in Atlantic salmon [11, 12, 18, 40, 62]. Importantly, the induction of antiviral genes observed in NI-HD also remained higher in *L. salmonis*-challenged fish fed GLs (I-HD, I-LD) five weeks post-infection.

A recent comparative transcriptomic study of pink, chum and Atlantic salmon found downregulation of antiviral immune genes in both the resistant (pink) and susceptible species (chum), thus drawing attention to other protective mechanisms in Pacific salmonids, centered around iron metabolism [16] and possibly availability of other nutrients. Highly diverse iron sequestration mechanisms appear to play a crucial role in resistance of pink salmon to *L. salmonis*, but iron withdrawal strategy in response to lice was also reported in Atlantic salmon [16]. This study suggested that modulation of this line of defense could be

Table 3 Examples of genes with differential expression in skin involved in apoptosis, stress responses, cytoskeleton and steroid and lipid metabolism (microarray data)

Gene	Abbreviation	Accession	NI-HD	I-C	I-LD	I-HD
Apoptosis and stress						
G0/G1 switch protein 2	*G0S2*	117545986	-1.41	1.24	-3.96	-4.28
Caspase 3A	*CASP3B*	S24639607	1.33	1.39	1.54	1.73
60 kDa heat shock protein, mitochondrial	*HSPD1*	223649223	1.39	1.50	1.86	1.97
Heat shock protein 4	*HSPA4B*	DY713457	1.38	1.19	1.69	1.97
Glutathione peroxidase type 2	*GPX1A*	CA345885	1.50	1.50	2.02	1.79
L-amino-acid oxidase		DY698830	2.54	1.05	1.31	1.34
Cytoskeleton						
Keratin 12		EG798776	1.28	1.80	1.66	2.38
Keratin type I cytoskeletal 17		DY692568	1.29	1.24	1.36	2.60
Type I keratin S8		CX357672	1.12	1.34	1.88	1.77
Type II keratin E3	*KRT4*	EG778421	1.09	1.27	1.48	2.57
Steroid and lipid metabolism						
Lipoprotein lipase	*LPL*	EG838215	-1.25	1.28	-1.97	-2.46
Fatty acid-binding protein, adipocyte	*FABP11A*	209735153	-1.19	1.29	-2.96	-3.01
Sex hormone-binding globulin beta	*GAS6*	DY699233	-2.00	1.23	-3.68	-5.64

Data are mean fold calculated from $\log_2$ (ER) values and compared to NI-C. Values with significant difference compared to NI-C group are underlined

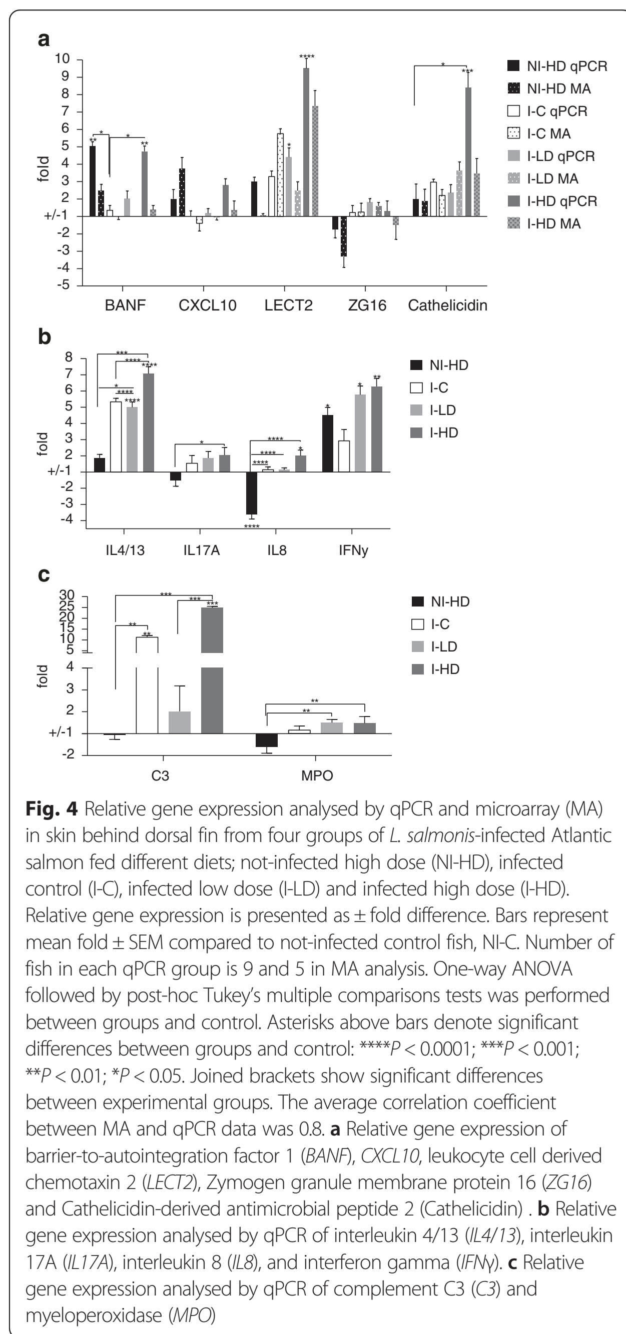


Fig. 4 Relative gene expression analysed by qPCR and microarray (MA) in skin behind dorsal fin from four groups of *L. salmonis*-infected Atlantic salmon fed different diets; not-infected high dose (NI-HD), infected control (I-C), infected low dose (I-LD) and infected high dose (I-HD). Relative gene expression is presented as ± fold difference. Bars represent mean fold ± SEM compared to not-infected control fish, NI-C. Number of fish in each qPCR group is 9 and 5 in MA analysis. One-way ANOVA followed by post-hoc Tukey's multiple comparisons tests was performed between groups and control. Asterisks above bars denote significant differences between groups and control: ****$P < 0.0001$; ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$. Joined brackets show significant differences between experimental groups. The average correlation coefficient between MA and qPCR data was 0.8. **a** Relative gene expression of barrier-to-autointegration factor 1 (*BANF*), *CXCL10*, leukocyte cell derived chemotaxin 2 (*LECT2*), Zymogen granule membrane protein 16 (*ZG16*) and Cathelicidin-derived antimicrobial peptide 2 (Cathelicidin) . **b** Relative gene expression analysed by qPCR of interleukin 4/13 (*IL4/13*), interleukin 17A (*IL17A*), interleukin 8 (*IL8*), and interferon gamma (*IFNγ*). **c** Relative gene expression analysed by qPCR of complement C3 (*C3*) and myeloperoxidase (*MPO*)

achieved by nutrition. In line with the proposal that sequestration of iron away from lice constitutes an aspect of protection, we observed increased expression levels of several genes coding for iron carrying and heme-binding proteins in groups exposed to GLs, including serotransferrin 1 and 2 in I-HD, and metalloreductase STEAP4 in I-LD (Fig. 2d, Table 2). In further support of this view, findings presented in a related paper (Stanko Skugor, personal communication) outlined the role of liver, muscle and distal kidney in iron sequestration in Atlantic salmon fed GLs-containing feeds.

qPCR analysis confirmed higher expression in I-LD and I-HD groups of several pro-inflammatory cytokines, chemokines and effectors, including the neutrophil attractant *LECT2* and cytokines *IFNγ* and *IL17A* involved in Th1 and Th17-guided immune responses in mammals (Fig. 4a, b). Preconditioning naïve salmon skin by feeding GLs appears to oppose suppression and modulation of host immunity by lice, as the lowest expression level of Type 1 genes was found in I-C fish at the end of the challenge trial (Fig. 2c). We also wish to draw the attention to context-dependent fine-tuning of skin responses by GLs, exemplified by *IL17A* and *MPO* regulation (Fig. 4b, c). Downregulation was observed in the not-infected group (NI-HD) and in contrast, high expression was found in GLs supplemented groups post-infection (I-LD and I-HD). This context-dependent regulation (in absence *vs* in presence of infection) indicates that the preconditioning by GLs acts at a level other than the effector/mediator molecules MPO and IL17A (e.g. at the level of sensors or adaptors). Additional studies are needed to understand this in more detail.

While induction of anti-inflammatory mediators and ECM components (e.g. collagens) involved in strengthening of the physical skin barrier characterised I-C group, the best-protected group (I-LD) showed the opposite response: highest induction of extracellular *MMPs* involved in digestion of collagens and other ECM proteins (Table 2). Marked upregulation of *MMPs* have been observed around lice attachment sites in Atlantic salmon [11, 17, 18, 40], and importantly, even more so in the resistant pink salmon 48 h post-infection [11]. Together with *MMPs*, a number of immune genes was most highly expressed in I-LD and I-HD groups. The group included cathelicidin antimicrobial peptide 2 (Table 2), previously implied in estrogen-mediated protection [39] and found to be responsive in stock bred for increased lice resistance [17], as well as two other genes coding for antimicrobial proteins, namely granzyme A and natterin-like protein. These findings suggest that host-interactions with or the skin microbiome *per se* could play a role in resistance to lice, which may open an exciting new field for future investigations. Serum amyloid is induced early in skin and spleen during *L. salmonis* infection in Atlantic salmon [40]. The relevance of serum amyloids as candidates of protection in this study (Fig. 2d) is underscored by observations of suppressed serum amyloid A in skin of susceptible species, and activation in resistant species [11, 12, 16]. Immune mediators might direct some of the observed changes in the group of genes governing tissue turnover of nutrients, e.g. expression of fatty acid-binding protein (Table 3), which was 4-fold higher in control compared to other infected groups, is regulated by Th2 cytokines IL4 and 13 in humans [63].

Co-regulation of genes encoding myofiber proteins and multiple glycolytic enzymes is a hallmark of transcriptomic changes observed in our previous studies [18, 38, 40], suggesting that contractions in lice infected skin are fueled by glycolytic oxidation of sugars. Suppression of contractile activity in GLs-fed groups implied in this study could be associated with protection against lice. Downregulation of a number of genes encoding lipid metabolism and tissue differentiation regulators and ECM components (Table 3) in fish exposed to GLs also deserves attention, as it likely affected composition and consequently physicochemical properties of skin and mucus. Dietary GLs treatment could result in skin and mucus becoming less nutritious for lice. Such changes could also interfere with the host recognition by *L. salmonis* and the ensuing fast attachment of parasites to skin. Lipids present in fish mucus play a significant role in defining its viscosity [64] with obvious consequences for the lice attachment process. Moreover, it has been known for a long time in mammals that lipid sebum extracts contain volatile host odor components involved in the attraction of parasites [65]; thus, changes in the lipid content or lipid composition might have the potential to affect retention/formation of host kairomone semi-chemicals in Atlantic salmon mucus. To what extent this plays a role in early or late stages of sea lice infection remains unknown, and additional studies are needed to better understand the underlying mechanisms.

Finally, the present study suggested that GLs-mediated suppression of sex hormone-binding globulin beta, involved in regulation of sex hormone levels [66], may have increased the availability of steroid hormones in protected fish (Table 3). The sex steroid hormonal system in fish skin is important in wound healing in sea bream [67] and is associated with protection against lice in Atlantic salmon [39]. Future feeding studies could explore the possibility to specifically promote beneficial expression of sex steroid hormones in skin while avoiding adverse hormonal effects in non-target tissues.

Conclusions

Feeding Atlantic salmon anti-attachment feeds containing GLs resulted in skewing of inflammatory responses towards Type 1 immunity, including gene expression programs centred on interferons in the skin prior to infection. Such dietary preconditioning seems beneficial upon encounter with the parasite as activation and maintenance of Type 1 immune genes coincided with the reduction in lice numbers. In addition, GLs-mediated gene expression changes implicated in the physicochemical properties of skin and mucus and metabolism of nutrients (iron, lipids and sugar) might interfere with the host recognition, attachment process and development of the parasite.

Data accessibility

Microarray gene expression data files have been deposited to Gene Expression Omnibus (GSE79393).

Additional file

Additional file 1: Table S1. Primers used for real-time qPCR analysis. **Table S2.** Formulation and proximate compositions of the feeds. (DOCX 23 kb)

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

HH, SW, AKB and SS designed and conducted the experiment. HH and SS carried out the molecular work, while HH, AK and SS analysed the data. HH and SS wrote the first draft, and all authors contributed to the final version of the manuscript. All authors read and approved the final manuscript.

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IV

Nutrigenomic effects of glucosinolates on liver, muscle and distal kidney in parasite-free and salmon louse-infected Atlantic salmon

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Abstract

Background: Reduction of *Lepeophtheirus salmonis* infection in Atlantic salmon achieved by glucosinolates (GLs) from *Brassica* plants was recently reported. However, wider application of functional feeds based on GLs requires better knowledge of their positive and adverse effects. **Methods:** Liver, distal kidney and muscle transcriptomes of salmon exposed to the extreme dose of GLs were profiled by microarray, while qPCR analysis followed up selected hepatic and renal responses under the extreme and moderate GLs dose during the *L. salmonis* challenge. Transcriptional analysis were complemented with measurements of organ indices, liver steatosis and plasma profiling, including indicators of cytolysis and bilirubin. Finally, the third trial was performed to quantify the effect of lower GLs doses on growth.

Results: The extreme GLs dose caused decrease in hepatic fat deposition, hepato-somatic index and growth, in line with microarray findings, which suggested tissue remodeling and reduction of cellular proliferation in the skeletal muscle and liver. Lower GLs inclusion levels in a follow-up trial did not show negative effects on growth. Microarray analysis of the distal kidney pointed to activation of anti-fibrotic responses under the overexposure. However, analyses of ALT, CK and AST enzymes in plasma provided no evidence of increased cytolysis and organ damage. Prevalent activation of phase-2 detoxification genes that occurred in all three tissues could be considered part of beneficial effects caused by the extreme dose of GLs. In addition, transcriptomic evidence suggested GLs-mediated iron and heme withdrawal response, including increased heme degradation in muscle (upregulation of *heme oxygenase-1*), decrease of its synthesis in liver (downregulation of *porphobilinogen deaminase*) and increased iron sequestration from blood (hepatic induction of *hepcidin-1* and renal induction of intracellular storage protein *ferritin*). This response could be advantageous for salmon upon encountering lice, which depend on the host for the provision of iron carrying heme. Most of the hepatic genes studied by qPCR showed similar expression levels in fish exposed to GLs, lice and their combination, while renal

induction of *leptin* suggested heightened stress by the combination of extreme dose of GLs and lice. The highest expression of *ifnγ* (cytokine considered organ-protective in mammalian kidney) was detected at the moderate GLs level. This fish also showed highest plasma bilirubin levels (degradation product of heme), and had lowest number of attached lice, further supporting hypothesis that making heme unavailable to lice could be part of an effective anti-parasitic strategy.
Conclusions: Modulation of detoxification and iron metabolism in Atlantic salmon tissues could be beneficial prior and during lice infestations. Investigation of anti-lice functional feeds based on low and moderate GLs inclusion levels thus deserves further attention.

Keywords: Atlantic salmon, *Salmo salar*, Salmon louse, *Lepeophtheirus salmonis*, Glucosinolates, Functional feeds, Iron, Antioxidant, Detoxification

Background

Despite significant attention given to finding alternative control strategies of the ectoparasite salmon louse *Lepeophtheirus salmonis*, the management of infections on salmon farms still heavily relies on the use of chemical treatments [1]. Several recent studies describe the severity of the situation in detail, including incurred economic losses [2], mortalities associated with application of chemical treatments [1] and in wild salmonid populations [3–5], risk of pathogenic virus transmission [6–8], and development of resistance to available parasiticides [9–12]. As chemical treatments are becoming limited and less efficient, there is an increasing interest in the development of anti-lice functional feeds.

Protection against lice can involve modulation of inflammation at the attachment site and induction of iron regulatory mechanisms [13–16], processes that can be modulated by diet [17, 18]. GLs constitute a heterogeneous family of sulfur-rich secondary plant metabolites occurring in cruciferous plants that are grown and consumed worldwide. Upon mechanical damage, GLs are hydrolyzed by the enzyme myrosinase into compounds that defend plants against a wide range of herbivores, including insects and aquatic invertebrates (reviewed by [19]). Ingested GLs are also hydrolyzed by the intestinal microflora [20]. Isothiocyanates (ITCs) constitute the major bioactive fraction of the hydrolysis products of GLs, with antibacterial properties *in vitro* [21], and antifungal effector properties in live plant cells [22].

62 GLs and related products are toxic to parasites in direct contact, but not much is known
63 about the mechanisms behind the avoidance behavior of chemical irritation. The ability to per-
64 ceive volatile ITCs has been well documented, especially in insects [23]. Avoidance and attract-
65 ant effects with non-host and host conditioned water, respectively, were shown in behavioral tests
66 *in vitro*, in a related, also parasitic louse species (*Caligus rogercresseyi*) [24]. An *in vivo* follow-
67 up study revealed activation of putative ionotropic receptor genes that could possibly be involved
68 in the olfaction and avoidance of salmon fed anti-lice feeds [25].
69 Currently, GLs and their breakdown products are attracting attention in fish nutrition re-
70 search because of their parasiticidal potential against the sea louse species that infect cultured
71 salmonids. Fish receiving GLs-based functional feeds could additionally benefit from their de-
72 toxifying and immunomodulatory properties. Modulation of cellular redox status appears to be
73 at the core of ITCs' bioactivity (reviewed in detail by [26]). The indirect antioxidant properties
74 of ITCs achieved through induction of phase-2 detox enzymes are considered responsible for
75 their anticancerogenic properties [27]. However, under certain conditions, ITCs can also induce
76 the pro-oxidant phase-1 enzymes [26]. The positive effects on human health of GLs and ITCs
77 present in diets rich in cruciferous vegetables were reported by several clinical studies [28–31].
78 However, GLs can exert anti-nutritional and toxic effects [32–36], and their wider use in aqua-
79 culture requires better knowledge of their actions. Reduced palatability and decreased growth are
80 among the main anti-nutritional effects of overexposure to GLs/ITCs in vertebrates, (reviewed in
81 [37]). When large quantities are ingested, the adverse health effects include deterioration of liver,
82 kidney and thyroid function [37].
83 Here we report findings from several feeding trials designed to investigate both the ben-
84 eficial and adverse effects of GLs, alone and during the *L. salmonis* challenge, on nutritional
85 parameters, gene expression and physiological responses in Atlantic salmon. In Trial 1, Atlantic
86 salmon not infected with lice (NI) but with an extreme inclusion level (13 %) of the GLs-con-
87 taining raw ingredient in their diet (NI-13) were compared to the control group with 0 % dietary
88 GLs (NI-C); hepatic, renal and muscle transcriptomes measured by microarrays were comple-
89 mented with measurements of the growth response, liver steatosis and plasma biochemistry. Trial
90 2 addressed the effects of the *L. salmonis* infection and GLs on growth, plasma biochemistry, and
91 gene expression of GLs-responsive candidates by qPCR in liver and distal kidney of infected (I)
92 fish exposed to control feed with 0 % GLs (I-C), medium (3.6 %) (I-3.6) and extreme level of

93 GLs (I-13). Finally, in Trial 3, a lower range of dietary GLs (0, 0.5, 1 and 2 %) were tested against
94 *L. salmonis* infection and growth, hepato-somatic and intestinal-somatic indices, liver steatosis
95 and muscle tissue composition were measured in I-C, I-0.5, I-1 and I-2 study groups.
96

97 # Results

98 **Fish growth**

99 In Trial 1, no significant reduction in growth was seen in parasite free NI-13 fish at the end of
100 the 17–18 day exposure period to the extreme dose of GLs (Table 1). After a longer exposure
101 period (47 days), significant (ANOVA: $F_{(2,177)} = 24.86$; I-C *vs* I-3.6: $P < 0.0001$; I-C *vs* I-13: $P <$
102 0.0001) growth reductions were observed in Trial 2 in I-13 and I-3.6 groups, 17 and 14 % lower
103 in comparison to I-C, respectively (Table 2). The calculated condition factor (CF) was found to
104 be very similar in NI-C and NI-13 (Table 1), but significantly (ANOVA: $F_{(2,177)} = 11.37$, I-C *vs*
105 I-3.6 $P < 0.0001$, I-C *vs* I-13 $P < 0.001$) different in Trial 2 (Table 2). The lower inclusion levels
106 in Trial 3 of the GLs-containing raw ingredient in I-0.5, I-1 and I-2 did not result in significant
107 differences in weight and CF between the dietary groups (Table 3). No differences in feed con-
108 sumption were found in any of the trials.
109

110 **Plasma profiling**

111 A basic panel of plasma tests was performed on parasite free fish from Trial 1 (NI-C and NI-13)
112 and lice infected fish from Trial 2 (I-C, I-3.6 and I-13) (Fig. 1). The elevated levels of profiled
113 enzymes in plasma are considered good indicators of cytolysis and cell leakage [38]. Alanine
114 aminotransferase (ALT) and aspartate aminotransferase (AST) are found in liver parenchymal
115 cells, and also in kidney, muscle and other tissues [38], while creatine kinase (CK) is an enzyme
116 that mainly increases due to leakage from muscle cells [39, 40]. No differences were found in
117 plasma levels of ALT, AST and CK between NI-C and NI-13. Interestingly, levels of all three
118 profiled enzymes were lower in Trial 2 during the lice infection in comparison to values measured
119 in lice free fish in Trial 1. The two trials are directly comparable as both took place under the
120 same conditions and at about the same time. Cholesterol levels were also significantly (*t*-test: $t_{(56)}$
121 $= 2.8$, $P = 0.007$) lower in infected fish than in fish not exposed to lice. Bilirubin, which is pre-
122 dominantly formed by the breakdown of heme present in hemoglobin [38], showed the highest
123 level in I-3.6 and was significantly different from the level measured in I-C and I-13 (Kruskal-

124 Wallis H-test: $\chi^2 = 5.99$, $df = 2$; I-C *vs* I-3.6: $P = 0.02$; I-13 *vs* I-3.6: $P = 0.05$). Stress causes ionic
125 imbalances in fish (see Djordjevic et al. [41] and references therein). We have previously seen
126 an increase in sodium (Na^+) ions in cortisol injected salmon during the lice challenge [42], hence
127 we measured Na^+ and potassium (K^+) plasma levels and calculated their ratio (Na/K) for each
128 group. Na^+ decreased in NI-13 fish while K^+ decreased in I-13, in comparison to their respective
129 controls (data not shown), while the Na/K was found to be highest in I-13.
130
131 **Liver steatosis**
132 Liver steatosis results from the three trials are shown in Fig. 2. A lowering effect of GLs on
133 steatosis was observed in all trials, while Trial 3 captured a smaller lowering effect of lice infec-
134 tion on liver steatosis.
135
136 **Organo-somatic indices and flesh quality**
137 In Trial 3, the hepato-somatic index (HSI) and intestinal-somatic index (ISI) were calculated
138 based on measurements taken from 10 fish from each of the study groups (Fig. 3). The HSI de-
139 clined as the inclusion of GLs increased, being significantly (Kruskal-Wallis H-test: $\chi^2 = 7.8$,
140 $df = 3$, $P = 0.008$) different between I-2 and I-C. GLs showed the opposite effect on ISI, with the
141 significant (ANOVA: $F_{(3,37)} = 3.8$, $P = 0.002$) difference observed between I-C and I-0.5 fish.
142 Flesh quality parameters were determined by the near infrared spectroscopy (NIR) per-
143 formed on Norwegian quality cut (NQC) samples (Table 4). Almost all of the significant changes
144 in the fatty acid profile were observed between I-0.5 fish and I-C, and I-2 and I-C.
145
146 **Microarray analyses**
147 Microarray analyses were performed on liver, distal kidney and muscle samples from the NI-C
148 and NI-13 fish from Trial 1. The criteria for differentially expressed genes (DEGs) were selected
149 by comparison of the test NI-13 group to NI-C, with log2-ER > 0.6 and $P < 0.05$. Magnitude of
150 diet-induced changes was similar in all tissues, while the number of DEGs that met our criteria
151 was highest in the liver (232), followed by the distal kidney (188) and the muscle (156).
152
153
154

155 **Liver**

156 Genes with roles in cell cycle and related processes (chromatin organisation regulation, DNA
157 replication and repair) comprised a large part of differentially expressed genes (Table 5). In-
158 creased expression was shown by several genes involved in the negative regulation of cellular
159 proliferation (e.g. *cullin 1b*, *btg1*, *abracl*), while a suite of genes coding for proteins required for
160 cell cycle, DNA replication and cellular division (e.g. *securin*, *condensin complex subunit 3* and
161 cyclins *G2/mitotic-specific cyclin-B1* and *cyclin-A2*) were downregulated. Activation of detoxi-
162 fication genes from both phase-1 (oxidation, reduction, and hydrolysis reactions) and phase-2
163 (conjugation reactions that increase water solubility of products generated by phase-1 enzymes)
164 pathways was observed (Table 5). Phase-1 monooxygenases *cytochrome P450 24A1* (*cyp24a1*)
165 may oxidise either xenobiotics or endogenous compounds. GLs-enriched diet also activated *epox-*
166 *ide hydrolase* (*ephx*) *1* and *ephx 2* that have roles in the protection from cyclic epoxides. Phase-
167 2 detox metabolism was represented by genes from well-known families responsive to ITCs in
168 mammals [43–46]: *UDP Glucuronosyltransferase 1 family polypeptide b7* (*ugt1b7*), *arylamine*
169 *N-acetyltransferase*, *pineal gland isozyme NAT-10* (*ary1*), involved in the detoxification of hy-
170 drazine and arylamine drugs, and *glutathione S-transferase theta* (*gstt*) *1* and *gstt3*. Stimulation
171 of biotransformation was in line with the slight induction of stress responses witnessed by the
172 upregulation of glucocorticoid receptor and *transcription factor jun b*. Expression of several other
173 genes with important metabolic roles was affected. The leader among the induced genes was
174 *CMP-sialic acid transporter* (Table 5) involved in the transfer of sialic acid into the Golgi lumen
175 where its conjugation to acceptor molecules takes place. *Pyruvate dehydrogenase kinase isozyme*
176 *2* (*pdk2*) is a master regulator of metabolic fluxes through the pathways of glucose and lipid
177 metabolism. Also of note was stimulation of bile salt export pump. The observed induction of
178 *hepcidin-1* and *cytochrome b reductase 1* (*cybrd1*), the key regulators of iron uptake, indicated a
179 decrease of plasma iron levels (Table 5). *Porphobilinogen deaminase*, involved in heme biosyn-
180 thesis, and *probable cytosolic iron-sulfur protein assembly protein ciao 1* (*ciao1*), key component
181 of the cytosolic iron-sulfur protein assembly complex, were downregulated. Dietary suppression
182 of a handful of immune genes was observed in the NI-13 group while a few negative regulators
183 of immune responses were found among upregulated genes (data not shown). In contrast, we
184 observed the hepatic induction of four complement system genes (Table 5).

185

186 **Distal kidney**

187 A suite of genes involved in xenobiotic metabolism were upregulated (Table 6). *NAD(P)H dehy-*
188 *drogenase quinone 1* (*nqo1*) is a highly-inducible gene coding for a multifunctional antioxidant
189 enzyme that is typically coordinately regulated with other detoxifying genes responsive to ITCs
190 [47]. Several genes that could serve as inhibitor of calcification and renal stone formation in-
191 cluded the upregulated *fetuin-A* [48] (Table 6) and *serine-pyruvate aminotransferase, mitochon-*
192 *drial* with double metabolic roles, gluconeogenesis in mitochondria and peroxisomal detoxifica-
193 tion of glyoxylate (Table 6). The latter function prevents calcium oxalate kidney stone formation
194 [49]. *Leptin*, which was induced by diet containing GLs, may contribute to the deterioration of
195 renal function through fostering proteinuria and TGFβ-mediated deposition of proteins in the
196 extracellular matrix (ECM) [50] (Table 6). Multiple indications support this claim. Two inhibi-
197 tors of TGFβ signalling were suppressed; *TGFβ-1-induced transcript 1 protein* that also regulates
198 Wnt pathway and *TGFβ receptor III* that could act as a decoy receptor involved in capturing and
199 retaining TGFβ [51]. Increased expression of *collagen a3(I)* was in parallel with downregulation
200 of *procollagen C-endopeptidase enhancer 1* that enhances collagen degradation. Upregulation of
201 *microfibrillar-associated protein 1*, component of the elastin-associated extracellular microfibrils
202 and *fibrinogen alpha chain* could contribute to deposition of extracellular insoluble fibrils, which
203 cause progressive renal dysfunction [52]. As could be expected, a group of genes that might play
204 protective roles against renal fibrosis was simultaneously activated by exposure to the high dose
205 of dietary GLs, including *ski-interacting protein* that inhibits TGFβ-mediated responses [53],
206 *Wnt-5b* that inhibits activation of the canonical (pro-fibrotic) Wnt pathway [54] and *matrix met-*
207 *alloproteinase 9* involved in digestion of ECM (Table 6). We also observed regulation of a num-
208 ber of immune genes (data not shown), some of which have known anti-fibrotic properties [55],
209 such as interferon γ (*ifnγ*) (Table 6) or might act as pro-fibrotic factors and contribute to tissue
210 damage. Regulation of iron metabolism was supported by the expression of two sideroflexins and
211 one ferritin gene (Table 6).

212

213 **Muscle**

214 Proapoptotic and inhibitory effects on proliferation in the muscle tissue were inferred from up-
215 regulated genes (Table 7), including *actin-related protein 2/3 complex subunit 1B*, involved in

216 the regulation of actin polymerization [56]; *Bax* that by antagonizing one of the apoptosis re-
217 pressors accelerates programmed cell death [57], and *androgen-induced proliferation inhibitor*
218 that plays a role in proliferative arrest [58]. Further support came from the downregulated *hepa-*
219 *rin-binding growth factor 1* that promotes cardiac hypertrophy and smooth muscle cell prolifer-
220 ation [59] (Table 7). Many genes with roles in diverse aspects of muscle-biology were regulated;
221 *myosin Va* has a role in actin filament-based movement [60]; *four and a half LIM domains protein*
222 *1* [61] has a role in muscle development or hypertrophy; *sodium/hydrogen exchanger* is involved
223 in muscle remodeling [62]; *tetranectin* is involved in muscle regeneration and muscle cell differ-
224 entiation [63]; *myotubularin* plays a role in skeletal muscle maintenance [64]; and *protein argi-*
225 *nine methyltransferase 5* is required for myogenesis and is also a positive modulator of insulin-
226 mediated glucose uptake in skeletal muscle cells [65]. Active remodeling of intracellular struc-
227 tures in muscle tissue was evidenced by upregulation of *stathmin*, which disrupts the microtubule
228 array [66] (Table 7). *Microtubule-associated protein 1 light chain 3 alpha* and *tripartite motif-*
229 *containing 55b* that plays regulatory roles in the myofibril assembly [67] were induced by the
230 GLs-enriched diet. The suppressed *aryl hydrocarbon receptor 2 beta* (*ahR2b*) indicated fine-
231 tuning of selected phase-1 and -2 cytochrome P450 isoforms, as has been described for the *ahR2b*
232 mammalian counterpart [68] (Table 7). *Glutathione transferase omega-1* (*gsto1*) with dual roles
233 in Ca-mediated muscle contraction, and cellular redox homeostasis as phase-2 biotransformation
234 enzyme [69] was also upregulated (Table 7). The gene *6-phosphogluconolactonase* (*pgls*), coding
235 for an enzyme required for the functioning of the pentose phosphate pathway when the rate of
236 oxidation of NADPH is accelerated [70], was also induced. Increased expression of *pgls* could
237 contribute to decreased lifetime of 6-phosphogluconolactone, its highly reactive and potentially
238 toxic substrate. *Alcohol dehydrogenase class-3* (*adh3*), also induced, constitutes the primary de-
239 fence mechanism against formaldehyde damage and may also indirectly mediate protection of
240 proteins against oxidation [71]. Gene encoding *heme oxygenase 1* (*ho-1*) that has important anti-
241 oxidant and cytoprotective activities was the most highly induced gene by dietary GLs in muscle
242 (Table 7). Previously, it was shown that ITCs-mediated induction of heme degrading HO-1 exerts
243 protective effects in kidney [72]. Together with activation of *uroporphyrinogen decarboxylase*,
244 involved in heme biosynthesis, these findings suggest increased turnover of heme under GLs
245 exposure.
246

247 **qPCR analyses**

248 qPCR analyses were used to validate microarray data and, in addition, compare responses of lice-
249 challenged salmon under the low (3.6 %) and extreme dose (13 %) of GLs to lice free and lice
250 infected fish given control feed. Three complement genes that were not measured by the micro-
251 array in liver were analyzed by qPCR (*c3*, *c5* and *c1qbp*), as several other genes of the comple-
252 ment system indicated complement activation in NI-13 (Table 5). Microarray results shown next
253 to qPCR results of NI-13 in Figs. 4, 5 (two first bars), revealed high concordance between the
254 two platforms. *Arylamine N-acetyltransferase (Ary1)*, involved in detoxification, and *comple-*
255 *ment factor H* (*cfh*) and *complement 3* (*c3*), involved in the complement immune response,
256 showed similar level of activation in lice challenged fish. *Cyp24a1*, a phase-1 detoxification gene
257 and a complement regulator *complement component 1Q binding* (*c1qbp*) were significantly
258 (*cyp24a1*: *t*-test: $t_{(15)} = 2.2$, $P = 0.04$; *C1qbp*: *t*-test: $t_{(15)} = 2.4$, $P = 0.03$) responsive to the high
259 dose of GLs without lice (NI-13). *Pyruvate dehydrogenase kinase isozyme 2* (*pdk2*) was signifi-
260 cantly induced only in I-C (*t*-test: $t_{(15)} = 3.121$, $P = 0.007$) group. *Tyrosine-degrading 4-hydrox-*
261 *yphenylpyruvate dioxygenase* (*hpd*) was the most highly induced gene in NI-13 on the distal kid-
262 ney microarray. Elevated levels of tyrosine in the absence of HPD activity are toxic to kidney
263 [73, 74]. qPCR analyses revealed most statistically significant (*t*-test: $t_{(15)} = 4.2$, $P = 0.0007$)
264 upregulation of *hpd* in I-3.6 salmon. I-13 group showed highest level of *solute carrier family 13*
265 *member 3-like* (*slc13a3*), while during infestation, *interferon* γ (*ifnγ*), and *integrator complex*
266 *subunit 7* (*ints7*) were most highly induced under the lower dose of GLs, in I-3.6 salmon. *Ifnγ*,
267 also highly induced in NI-13, is protective against renal injury induced by arsenite by modulation
268 of detoxification pathways [75] and experimental renal fibrosis following chemotherapeutic ex-
269 posure, explained by increasing the viability of renal tubular cells [76]. *Slc13a3* is highly ex-
270 pressed on the basolateral membrane of proximal kidney tubule cells, contributes to heavy metal
271 detoxification [77, 78] and is involved in the selective uptake of Krebs cycle intermediates [77].
272 *Ints7* plays a role in the DNA damage response pathway that typically results in cell cycle arrest
273 [79]. qPCR analysis revealed appreciable induction of *leptin* in I-13 (6-fold compared to NI-C)
274 while I-C and I-3.6 groups showed slight downregulation. The knockout of *abhydrolase domain-*
275 *containing protein 6* (*abhd6*), which was significantly (*t*-test NI-C *vs* I-13: $t_{(15)} = 2.1$, $P = 0.05$;
276 I-C *vs* I-13: $t_{(16)} = 3.1$, $P = 0.074$) induced in I-13 fish, results in downregulation of genes involved
277 in *de novo* fatty acid synthesis and lipogenesis in murine kidney [80].

278 **Discussion**

279 The interest for GLs and their breakdown products in Atlantic salmon aquaculture lies in their
280 parasiticidal potential against salmon louse alongside beneficial effects related to the improve-
281 ment of the antioxidant status and detoxification abilities. However, based on knowledge from
282 vertebrate studies, both adverse and positive effects of dietary GLs could be anticipated. An un-
283 desired consequence, most pronounced at the extreme dietary level of GLs, was the observed
284 reduction in growth seen in Trial 2. Microarray profiling proposed molecular players behind the
285 reduction in growth mediated by high levels of dietary GLs. Higher mRNA levels in NI-13 in
286 comparison to control were seen for a number of genes involved in the negative regulation of
287 proliferation in both liver and muscle. This was in line with a number of suppressed genes with
288 roles in the development, maintenance and hypertrophy of muscle in the transcriptome of fish
289 under the extreme exposure to GLs. In contrast, the negative effect on growth was not shown in
290 Trial 3, with up to 2 % of the GLs-containing raw ingredient included in the feed. With respect
291 to other potential beneficial effects of GLs, of note was the reduction of liver steatosis (Fig. 2a)
292 measured even at a low level of GLs (I-2) and decrease of HSI in I-2 (Fig. 3a). In Trial 3, an
293 increase in ISI with the increasing level of GLs was revealed, being highest and significant at the
294 lowest inclusion level of GLs (Fig. 3b). Interestingly, fillet quality traits profiled by NIR for I-
295 0.5, I-1 and I-2 fish revealed minor differences (Table 4).

296 Microarrays of the distal kidney of NI-13 was characterized by the concerted activation
297 of DNA damage response genes (Table 6), and suggested activation of anti-fibrotic responses
298 and those implied in the prevention of renal stone formation. This was not reflected in the level
299 of plasma indicators of tissue damage (ALT, AST and CK) in Trial 1 (Fig. 1). In fact, most had
300 lower values in NI-13 compared to NI-C. Furthermore, reduced enzyme levels in fish from Trial
301 2 in comparison to fish from Trial 1 were likely the reflection of lowered tissue metabolic activity
302 during the lice challenge, possibly mostly affecting muscle, as judged by the drop in CK levels.
303 Nevertheless, evidence produced with qPCR, particularly *leptin* data (Fig. 5), pointed out that I-
304 13 fish stand an increased risk of developing renal pathophysiology in case of prolonged simul-
305 taneous exposure to high levels of the two stressors. The increase of the Na/K ratio in I-13 (Fig.
306 1) could be seen as a warning sign that suggested adverse alteration of the hydromineral balance
307 in this fish in comparison to I-C. However, the high expression level of *leptin* and *abhd6* seen in
308 I-13 (Fig. 5) completely diminished in I-3.6, and moreover, the moderate level of GLs promoted

309 the expression of *ints7* and *ifnγ*, the latter of which has numerous documented protective roles in
310 the mammalian kidney.

311 Expectedly, GLs stimulated the expression of genes involved in detoxification. In contrast
312 to the long-held notion that GLs-derived ITCs selectively activate phase-2 while suppressing
313 phase-1 detoxification pathways [26, 81], our data revealed activation of a diverse group of genes
314 related to both pathways. In addition to the well-documented antioxidant properties of ITCs re-
315 lated to induction of phase-2 enzymes [26], their pro-oxidant properties are likely related to the
316 simultaneous induction of phase-1 enzymes. Phase-1 detoxification is dominated by reactions
317 involving cytochrome P450 enzymes, which are abundantly present in the endoplasmic reticulum
318 in liver and kidney. Hepatic induction of *cyp24a1* and two epoxide hydrolases (*ephx1* and *2*) from
319 Phase-1 (Table 5) occurred in parallel with the suppression of *ephx2* in kidney (Table 6), and
320 *cytochrome p450 1a1* and *ahR2b*, which regulates P450 enzymes, in muscle (Table 7). Activation
321 of genes from phase-2 metabolism was prevalent in all three tissues. The extreme GLs-enriched
322 diet induced key genes required for glutathione (GSH) based detoxification processes that result
323 in formation of water-soluble products that can be easily excreted. *Glutamate-cysteine ligase*
324 *catalytic subunit* encoding the first rate-limiting enzyme of glutathione synthesis was upregulated
325 in distal kidney (Table 6) while glutathione transferases that catalase conjugation of GSH to xe-
326 nobiotic products of phase-1 detoxification steps, were induced in liver (*gstt1* and *gstt3*) (Table
327 5) and muscle (*gsto1*) (Table 7). Furthermore, observed increase in the expression of *nqo1* in
328 distal kidney (Table 6), *ary1* and *ugt1b7* in liver (Table 5), and *pgls* and *adh3* in muscle (Table
329 7), may all contribute to the better protection against xenobiotics in fish exposed to dietary GLs.
330 Another potentially important effect of GLs is regulation of iron metabolism in all three tissues.
331 Levels of bioavailable iron are determined by intestinal absorption and macrophage recycling of
332 iron from hemoglobin. *Hepcidin-1* (*hepc1*) is a liver peptide that modulates intestinal iron ab-
333 sorption and acts to attenuate iron release from tissue macrophages and hepatocytes. Hepatic
334 induction of *hepc1* and *cybrd1* (Table 5) in fish exposed to GLs could result in increased iron
335 sequestration in liver, thus lowering iron plasma levels. Most of the intracellular iron is used in
336 mitochondria for heme biosynthesis or in cytoplasm for the assembly of iron-sulfur clusters that
337 are incorporated into extra-mitochondrial iron/sulfur containing proteins. Cellular iron status de-
338 termines the extent of iron-sulfur cluster assembly and thereby regulates expression of genes for

339 iron storage, transport, and utilization. Concomitant downregulation of *porphobilinogen deami-*
340 *nase*, involved in heme biosynthesis provided solid indication that iron excess stimulates cyto-
341 solic FeS cluster biogenesis (Table 5). Renal induction of *ferritin*, involved in iron sequestration
342 within cells, additionally supported possibility that the access of iron to circulation was reduced
343 by GLs (Table 6). Furthermore, the most highly induced gene in muscle was *ho-1* (Table 7), an
344 enzyme with the key role in degradation of heme into iron and biliverdin, which is then converted
345 to bilirubin [82]. Iron tissue dynamics within salmonid hosts are believed to play an important
346 role in the outcome of lice infections [16]. Coordinated and early changes in the expression of
347 genes involved in metabolism of iron and erythropoiesis in spleen, head kidney and liver were
348 seen in lice-infected Atlantic salmon [83, 84]. The resistant pink salmon showed highly diverse
349 iron sequestration and homeostasis mechanisms, including an early upregulation in the head kid-
350 ney of *hepc1*, *ho-1* and several genes involved in iron tissue storage and sequestering of iron from
351 blood [16]. *Bilirubin* that is predominantly formed by breakdown of heme present in hemoglobin
352 showed highest level in the best-protected infected group (I-3.6, Fig. 1). The observed increase
353 in *bilirubin* was likely not caused by liver damage, as levels of ALT and AST went down during
354 the lice infection. With respect to protection against the parasite, of note are also decreased levels
355 of cholesterol in all infected dietary groups in comparison to NI fish. Cholesterol deprivation of
356 lice by the infected host can limit their growth. This was also implied in our recent study where
357 estrogen-mediated protection was associated with a regulation of skin genes involved in choles-
358 terol metabolism, among several other potentially beneficial processes [18]. *Lepeophtheirus*
359 *salmonis*, which is an obligate parasite, lacks genes required for the cholesterol biosynthesis en-
360 coded in its genome (Prof. Frank Nilsen, personal communication).

361 Profiling of skin of lice-infected fish from the GLs feeding trial revealed reduced number
362 of attached lice and massive activation of antiviral responses, likely including IFN-mediated re-
363 sponses [17]. The type and magnitude of immune responses at the site of parasite attachment in
364 skin [13, 15] and in internal immune organs [85] contribute to susceptibility to *L. salmonis* in
365 Atlantic salmon. Several complement system genes responded to GLs in NI-13 – this type of
366 immune system preconditioning by diet could be helpful upon parasite encounter.

367

368

369

370 ## Conclusions

371 Our findings encourage future use of GLs-based feeds due to their beneficial effects on the ex-
372 pression of genes with detoxifying and iron-regulatory roles in multiple fish tissues. The further
373 refinement of anti-lice functional feeds will require understanding of how the beneficial processes
374 can be promoted to achieve protection against lice while not decreasing growth or posing any
375 adverse effects on tissue functions.

376

377 ## Methods

378 ### Preparation of feeds and fish trials, production of feeds and copepodids

379 The trials were approved by the National Animal Research Authority, in line with the "European
380 Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific
381 purposes" were performed at Ewos Innovation in Dirdal, Norway. Feeds with various inclusion
382 levels of glucosinolates (GLs) were produced at the Ewos Innovation plant in Dirdal, Norway.
383 The GLs were added to feeds by spraying the powdered raw ingredient onto the base pellet under
384 vacuum conditions. Air pressure was then allowed to return to normal and the GLs-containing
385 powder was sucked into the core of the pellet. All diets had a pellet size of 5 mm. An overview
386 of trials, harvested samples and applied analysis methods are shown in Additional file 1: Table
387 S1. The study groups were denoted by trial (Trial 1 and Trial 2), treatment (not infected - NI or
388 infected - I) and the level of ingredient (0 % - C, 3.6 or 13 % inclusion level).

389 The graphical overview of Trial 1 and Trial 2 is shown in Fig. 6. Fish in six tanks with 18
390 fish in each were fed the 0 % GLs control feed for one month (acclimation) before separation of
391 fish into two groups (Trial 1). Fish in three tanks continued on the control feed (NI-C) while fish
392 in three other tanks were given the 13 % GLs feed (NI-13) during 17–18 days. At the end of this
393 period, weight and length measurements (n = 18 in each group) and samples of liver, muscle and
394 distal kidney (n = 9 in each group) were taken. Tissue samples were placed in RNA*later* (Am-
395 bion®, Austin, TX, USA) and stored at 4 °C for 24 h and then stored at -80 °C until further
396 analyses. In addition, 5 liver samples from each group were placed in neutral buffered formalin
397 for histology. In Trial 2, fish in tank triplicates received control diet, and diets enriched with 3.6
398 and 13 % of the GLs-containing raw ingredient during three weeks. There were 90 fish in each
399 feed group (control, 3.6 and 13). The amount of uneaten pellets was recorded weekly to calculate
400 the feed intake for each of the feeds over three weeks. After this, all 15 tanks were given a control

401 feed (EWOS Opal) for 10 days, and number of fish in each group was reduced to 60. This was
402 followed by feeding fish (n = 20 in each tank) control feed, 3.6 and 13 % GLs enriched feeds in
403 tank triplicates for 12 days (pre-infection period), and throughout the 31–35 day period of *L.*
404 *salmonis* infection (post-infection period). During a sampling period of 4 days; number, stage
405 and gender of lice on each fish were recorded. Furthermore, liver and distal kidney tissues were
406 sampled in RNA*later* from 9 fish in each group. In addition, 4–6 liver samples from each group
407 were placed in neutral buffered formalin for histology. The treatment groups tested in this part
408 were named: infected control (I-C), infected 3.6 (I-3.6) and infected 13 (I-13). Fulton's condition
409 factor was calculated by the formula: ($100\ BWFL^{-3}$) [86] in both Trial 1 and Trial 2. Finally,
410 peripheral blood from the caudal vein was collected into heparinized vacutainers from fish in
411 each group in Trial 1 (n = 15–16) and Trial 2 (n = 9). Fish tanks used in both trials were 500 l
412 circular flow-through tanks with an average temperature of 8.7 °C and 27.4 ppt salinity.
413 Trial 3 was performed in August 2013 with low inclusion levels of the GLs-containing
414 raw ingredient (0, 0.5, 1 and 2 %). Groups in this trial were thus infected control (I-C), infected
415 0.5 % (I-0.5), infected 1 % (I-1) and infected 2 % (I-2) and each group of fish was allocated into
416 three tank replicates with 23–26 fish in each tank. Tank conditions, feeding regime and infection
417 of fish with copepodids were similar to the infection study in Trial 1 and 2 [17], but fish were fed
418 the experimental diets for 23 days before lice challenge, and sampling was performed after 26–
419 28 days of infection. At this time point, Norwegian Quality Cut (NQC) samples were harvested
420 from 10 fish from each group according to the standard procedure. Liver samples (4–6) from
421 each group were placed in neutral buffered formalin for histology. In addition, hepato-somatic
422 and intestinal-somatic indices were calculated from 10 fish from each group, performed by
423 weighing the liver and intestinal mass of each fish respectively and dividing this value by the fish
424 weight.
425 Statistical differences of weights, CFs and organosomatic indices were assessed by One-
426 way ANOVA with subsequent Tukey's multiple comparisons test in the GraphPad Prism Soft-
427 ware 6.0 as criteria for Gaussian distribution were met by Shapiro-Wilkes test and in addition the
428 equal variance test (Brown-Forsythe). Students t-test was used for analyzing weights and CF in
429 Trial 1. The level of significance for all analyses was set at $P < 0.05$.
430
431

432 **Challenge with salmon lice**

433 Challenge trials were performed as described in [17]. In short, the lice population used in Trial 2
434 and 3 originated from a nearby location (Oltesvik), and were maintained on Atlantic salmon hosts
435 kept in 850 l circular flow tanks at the Ewos Innovation salmon lice lab. Before infection, the
436 copepodid density was measured in a zooplankton counting chamber, where at least 4 samples
437 were counted to ensure accuracy of estimation. Experimental infection was performed with 50
438 copepodids per fish. During the tissue sampling period of 4 days, when most lice had reached the
439 preadult stages, recordings of the exact number, stage and gender of lice on each fish were made.

440

441 **RNA extraction and gene expression analysis**

442 ***RNA extraction***

443 Nine tissues samples of approximately 5 × 5 mm in size were excised from the fish and put in
444 RNAlater at 4 °C and then to 80 °C until further use. Total RNA extraction was performed by
445 the RNeasy Mini Kit (Qiagen) and Trizol (GIBCO, Life Technologies). In brief, Trizol (GIBCO,
446 Life Technologies, Carlsbad, CA, USA), zirconium oxide beads (VWR, Oslo, Norway) and ap-
447 proximately 50 mg of tissue was homogenized in FastPrep-24 homogenizer (MP Biomedicals,
448 Santa Ana, CA, USA). Chloroform was added to separate RNA into the supernatant, which was
449 subsequently run through the RNAeasy Mini Kit clean-up procedure provided by Qiagen (Hilden,
450 Germany). The RNA was diluted with 30 µl of RNAse free H_2O, concentration was determined
451 by spectrophotometry using NanoDrop ND1000 (Nanodrop Technologies, Wilmington, DE,
452 USA) and stored at -80 °C. Integrity of RNA was assessed with Agilent 2100 BioAnalyzer (Ag-
453 ilent, Santa Clara, CA, US) and RNA Nano kits, and only samples with RNA integrity number
454 (RIN) of 8 or higher were used for microarray.

455

456 ***Microarray analyses***

457 All reagents used in the microarray procedure were from Agilent Technologies. Liver, distal kid-
458 ney and muscle samples ($n = 5$) from fish from each group (NI-C, NI-13) in Trial 1 were analyzed
459 by microarray, and compared to pooled controls of two fish from each diet from the same organ.
460 The test samples and pooled controls were labelled with respectively Cy5 and Cy3, 100 ng of
461 RNA per reaction, by using The Two-colour Quick Amp Labelling kits and Gene Expression
462 Hybridization kits. The hybridization step lasted 17 hours at 65 °C with rotation speed 10 rpm,

463 followed by immersion for one minute each in Gene Expression Wash Buffer I at room temper-
464 ature and subsequently washing in Gene Expression Wash Buffer II at 37 °C. By scanning slides
465 using GenePix Personal 4100A scanner with 5 um resolution and manually adjusted laser power,
466 an equal intensity ratio between Cy3 and Cy5 channels with minimal oversaturation was
467 achieved. GenePix pro software 6.0 was used for feature extraction, assessment of spot quality,
468 and spot-grid alignment. Low quality spots were flagged by the software, and Lowess normali-
469 zation of log2-expression ratios (ER) was performed. Differentially expressed genes (DEGs)
470 were selected by comparison with not infected control (NI-C): log2-ER > 0.6 and $P < 0.05$ in at
471 least one group. Nofima's bioinformatics system (STARS) [87] was used for data analyses.
472

473 ***cDNA synthesis and qPCR protocol***

474 For qPCR analysis, RNA from 9 fish from the Trial 1 groups; NI-C, NI-13, and Trial 2 groups;
475 I-3.6, I-13 and I-C, were used. NI-C from Trial 1 was deemed as an appropriate control for fish
476 in Trial 2 as both trials took place under the same environmental conditions in the Salmon Lice
477 Lab in Dirdal and with negligible time difference; less than a week passed between the two sam-
478 plings. RNA was reverse transcribed to cDNA by using the cDNA Affinity Script (Agilent Tech-
479 nologies, Matriks AS, Oslo, Norway) and protocol provided by the manufacturer. Each reaction
480 consisted of 3 µg RNA, 1 µl of random primers and 2 µl of oligo DT primers. The synthesized
481 cDNA was diluted 10 times and stored at -20 °C until further use. The qPCR reactions were run
482 in duplicates. Each reaction (12 µl) contained 4 µl of cDNA, 10 µM primers and SYBR Green I
483 Master mix (Roche); analyses were run in LightCycler 480 in 96 well plates. Published gene
484 sequences were used to design primers (Additional file 1: Table S3) for quantitative Real Time
485 PCR (qPCR) reactions by CLC Workbench software. Cycling conditions in LightCycler 480 in-
486 strument (Roche, Applied Science) were 5 min denaturation step at 95 °C, 40 cycles of denatur-
487 ation (10 s at 95 °C), annealing (20 s at 60 °C) and extension (15 s at 72 °C), followed by melting
488 curve analysis with measurements of the fluorescence was performed in the temperature range
489 between 65–97 °C. The crossing point value was found by using the maximum-second-derivative
490 method (Roche diagnostics), followed by the -ΔΔCt method with comparison to reference gene
491 *elongation factor 1 alpha* (*ef1α*) to find the relative expression of target genes. No signs of gDNA
492 contamination were found by running a subset of RNA samples together with *ef1a* and
493 SybrGreen. Specificity and efficiency were confirmed by melting curve analysis, agarose gel

494 electrophoresis and two-fold serial dilutions of cDNA for each primer pair in triplicates. PCR
495 efficiency of all genes ranged from 1.96–2. Data were analyzed with students *t*-test in the
496 GraphPad Prism Software 6.0 if criteria for Gaussian distribution were met by Shapiro-Wilkes
497 test. All qPCR data showed equal variance by the Brown-Forsythe test. If criteria for normality
498 were not met, the Mann-Whitney test was used. The level of significance for all analyses was set
499 at $P < 0.05$.
500
501 **Histology**
502 Four to six fish selected randomly from each group in Trial 1; NI-C and NI-13, Trial 2; I-C, I-3.6
503 and I-13, and Trial 3; I-2 group, NI-C and I-C were subjected to histological analysis. Liver
504 samples were fixed in neutral buffered formalin for 48 h with the change of formalin after 24 h
505 followed by dehydration, paraffin-embedding, sectioning and haematoxylin and eosin (HE) stain-
506 ing by standard histological procedures. The 4 µm blinded sections were examined with a Leica
507 DFC 420 microscope equipped with a digital imaging system (Leica Image Analysis). Steatosis
508 scoring was performed by studying five representative fields at 20× original magnifications, se-
509 lected randomly. The areas were scored for microvesicular and macrovesicular steatosis follow-
510 ing the scoring system and method described in [88] and in Additional file 1: Table S2. A pro-
511 portion of the liver samples were also stained with Periodic-Acid Schiff (PAS) to exclude glyco-
512 gen accumulation as a cause of vacuole formation.
513 **Blood plasma profiling**
514 Blood plasma profiling was performed on 15 individuals from NI-C and 16 individuals from NI-
515 13 in Trial 1, and 9 fish from each of the groups in Trial 2. The full automatic Adria 1800 system
516 in the Central clinical laboratory at the Norwegian University of Life Sciences was used to meas-
517 ure a basic panel of plasma parameters, including ALT (alanine aminotransferase), AST (Aspar-
518 tate aminotransferase), CK (Creatine Kinase), Cholesterol, Na (sodium), K (potassium) and bili-
519 rubin. All parameters except the ions were found by measuring the optical density at a given
520 absorbance. Na and K levels were assessed by the indirect potentiometric procedure. Significant
521 differences for each plasma parameter were analyzed between Trial 1 and Trial 2, treating the
522 groups in each trial as one. Significant differences between were also assessed between the groups
523 in Trial 1 and Trial 2, separately. Data were analyzed by Student's *t*-test or One-way ANOVA
524 with subsequent Tukey's multiple comparisons test in the GraphPad Prism Software 6.0 if criteria

525 for Gaussian distribution were met by Shapiro-Wilkes test. All parameters showed equal variance
526 in Brown-Forsythe test. If criteria for normality were not met, the Mann-Whitney or the Kruskal-
527 Wallis test was used followed by the *post-hoc* Dunn's test. The level of significance for all anal-
528 yses was set at $P < 0.05$.
529

530 **Near infrared spectroscopy**

531 Near infrared spectroscopy (NIR) is a spectral method based on the fact that different feed com-
532 ponents have characteristic NIR absorption bands when exposed to specific wavelengths of in-
533 frared light. NIR analysis was performed on NQC samples from 10 individuals from each group
534 from Trial 3 by using the NIR XDS system (Foss, Hillerød, Denmark) at Ewos Innovation, Dir-
535 dal. NIR calibration equations were found beforehand by analyzing 1300 NQC samples of fish
536 ranging in size from 0.1 to 6 kg. Reference values were based on well-established internal and
537 external sources. Individual NQC samples were thoroughly grinded in a meat grinder shortly
538 after slaughter. The groups thus analyzed were I-0.5, I-1 and I-2 in addition to I-C. The levels of
539 ash, energy, fat, moisture, phosphorous, protein, pigment, total monosaturated fatty acids, and a
540 range of fatty acids (PUFA) including: total n-3 polyunsaturated fatty acids (PUFA), Total n-
541 6PUFA, total PUFA, 14:0, 16:0, 16:1, 18:0, 18:1, 18:2n-6, 18:3n-3, 18:3n-6, 18:4n-3, 20:1,
542 20:3n-3, 20:3n-6, 20:4n-3, 20:4n-6, 20:5n-3, 22:1, 22:4n-6, 22:5n-3, 22:5n-6, 22:6n-3 were ana-
543 lyzed. Statistical differences were assessed by One-way ANOVA with subsequent Tukey's mul-
544 tiple comparisons test in the GraphPad Prism Software 6.0 if criteria for Gaussian distribution
545 were met by the Shapiro-Wilkes test and in addition the equal variance test (Brown-Forsythe). If
546 criteria for normality were not met, the Kruskal-Wallis test was used followed by the *post-hoc*
547 Dunn's test. The level of significance for all analyses was set at $P < 0.05$.
548

549 **Additional file**

550 **Additional file 1: Table S1.** An overview of samples and analysis methods applied in Trial 1, 2
551 and 3. **Table S2.** Scoring of liver steatosis in individual sections based on [88]. **Table S3.** Primer
552 list

553 **Abbreviations:** *abhd6: abhydrolase domain-containing protein* 6; *adh3*: *Alcohol dehydrogenase*
554 *class-3*; ALT: alanine aminotransferase; *ary1*: *arylamine N-acetyltransferase, pineal gland iso-*
555 *zyme NAT-10*; *ahr2b*: *aryl hydrocarbon receptor 2 beta*; *AST*: aspartate aminotransferase; *cfh*:

556 *complement factor H*, *c1qbp*: *complement component 1Q binding*, *c3*: *complement 3*; *c5*: *com-*
557 *plement 5*; CK: creatinine kinase; *cybrd1*: cytochrome b reductase 1; cyp24a1: *cytochrome P450*
558 *24A1*; DEG: differentially expressed genes; ECM: extracellular matrix; *ephx*: *epoxide hydrolase*;
559 GLs: Glucosinolates; *gstt*: *glutathione S-transferase theta*; *gsto1*: *Glutathione transferase*
560 *omega-1*; *ho-1*: *heme oxygenase 1*; HIS: hepato-somatic index; I-C: Infected control; I-0.5: In-
561 fected control fed 0.5 % inclusion level of GLs; I-1: Infected control fed 1 % inclusion level of
562 GLs; I-2: Infected control fed 2 % inclusion level of GLs; I-3.6: Infected control fed 3.6 % inclu-
563 sion level of GLs; I-13: Infected control fed 13 % inclusion level of GLs; *ifnγ*: *interferon gamma*;
564 *ints7: integrator complex subunit 7*; ISI: intestinal-somatic index; ITC: *isothiocyanate*; K: potas-
565 sium; *L. salmonis*: *Lepeophtheirus salmonis*; Log2-ER: log2-Expression Ratios; MA: microar-
566 ray; Na: sodium; *nqo1*: *NAD(P)H dehydrogenase quinone 1*; NI-C: Not infected control; NI-13:
567 Not infected fed 13 % inclusion level of GL; NIR: near infrared spectroscopy; NQC: Norwegian
568 quality cut; *ciao1*: *probable cytosolic iron-sulfur protein assembly protein ciao 1*; *pdk2*: *pyruvate*
569 *dehydrogenase kinase isozyme 2*; *pgls*: *6-phosphogluconolactonase*; qPCR: quantitative PCR;
570 SEM: standard error of the mean; *slc13a3*: *solute carrier family 13 member 3-like*; *tgfβ*: *trans-*
571 *forming growth factor beta*; *hpd*: *tyrosine-degrading 4-hydroxyphenylpyruvate dioxygenase*;
572 *ugt1b7*: *UDP Glucuronosyltransferase 1 family polypeptide b7.*
573
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817 1545.

818

819 **Declarations**

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823

824 **Ethics approval and consent to participate**

825 The experimental facilities used in this study at Ewos Innovation, Dirdal, Norway, number 131
826 was approved by the Norwegian Animal Research Authority 02.02.2012 until 25.01.16. The ex-
827 periments/procedures have been conducted in accordance with the laws and regulations control-
828 ling experiments/procedures in live animals in Norway, e.g. the Animal Welfare Act of 20th
829 December 1974, No 73, chapter VI sections 20–22 and the Regulation on Animal Experimenta-
830 tion of 15th January 1996.

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832

Consent for publications

Not applicable.

Availability of data and material

The datasets supporting the conclusions of this article are included within the article and its additional files. Microarray gene expression data files have been deposited to Gene Expression Omnibus (GEO) under accession number GSE84288.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

AKB, HH, JP, SS, OE and SW were all involved in designing the experiments. JP and SW designed the feeds. AKB produced copepodids, conducted the experiment and counted lice at the end of the challenge trial, while HH and SS sampled the tissues in all 3 trials. HH and SS carried out the microarray lab work, and work on organo-somatic indices, NQC profiling and plasma data analysis. SS performed and analyzed qPCRs. AK, HH and SS analyzed the gene expression data. HH performed and described the histology data while OE supervised the analysis. SS wrote the first draft, and all authors contributed to the final version of the manuscript. All authors read and approved the final version of the manuscript.

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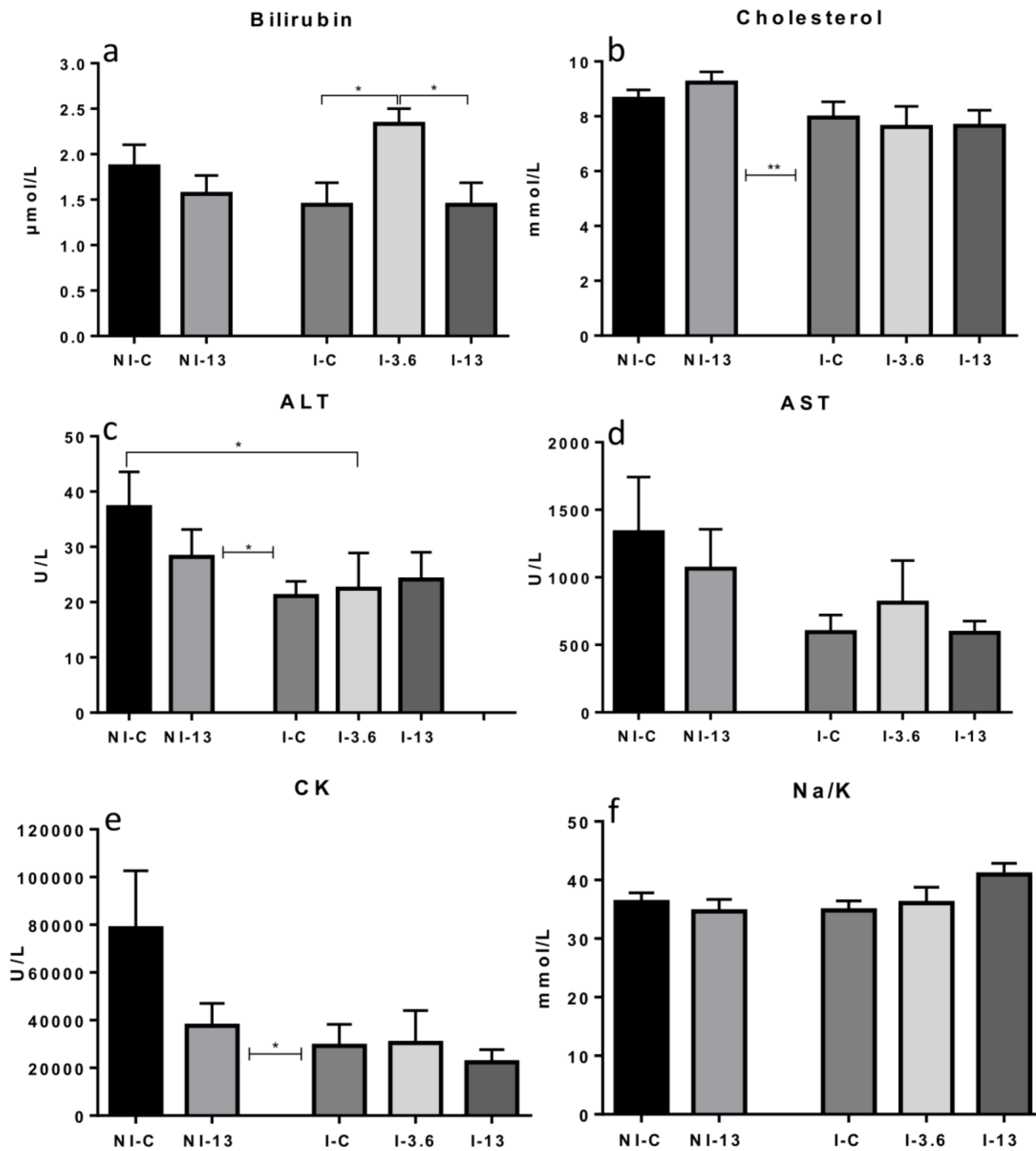
864 **Figures**

865

866 **Fig. 1**



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868 **Fig. 1** Blood plasma levels of total bilirubin (**a**), cholesterol (**b**), alanine aminotransferase (ALT)

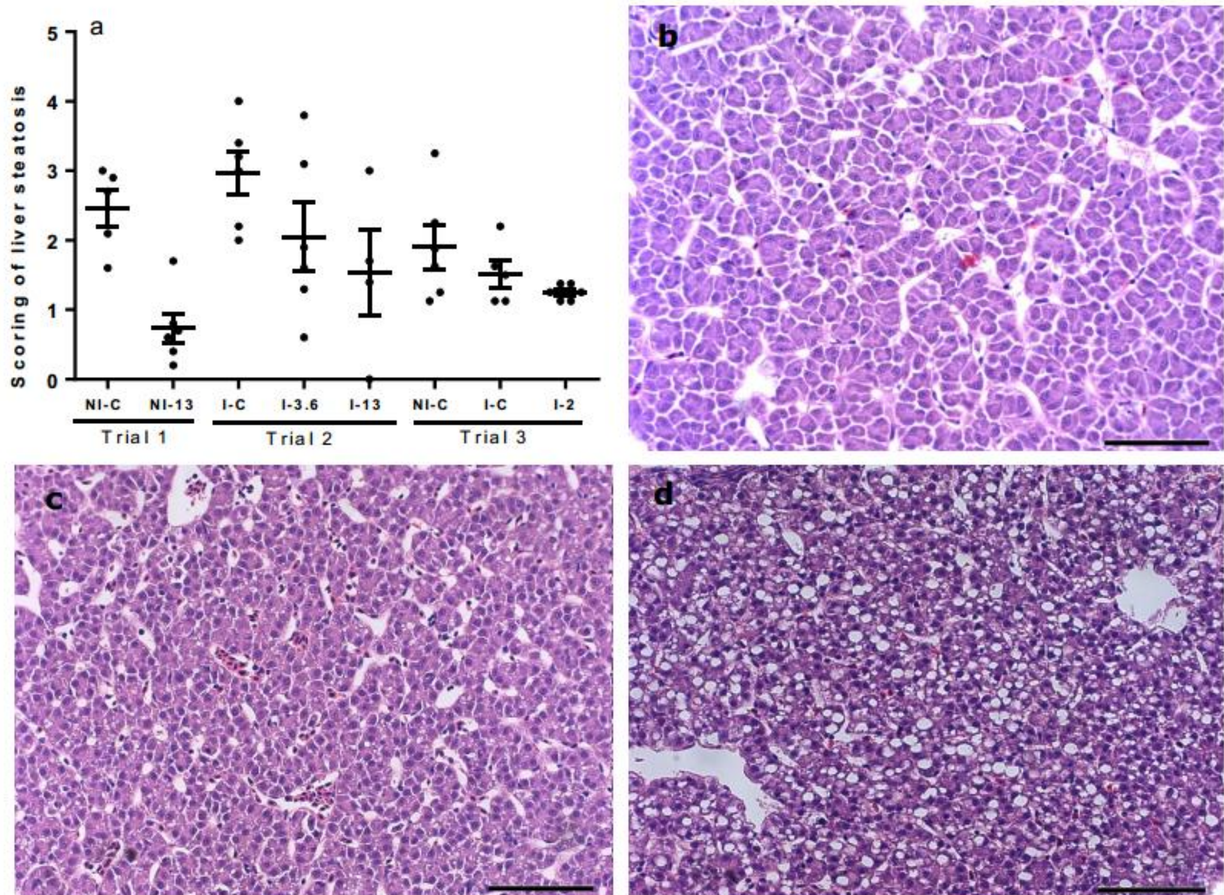
869 (**c**), aspartate aminotransferase (AST) (**d**), creatine kinase (CK) (**e**) and sodium (Na^+) to potas-

870 sium (K^+) ratio (Na/K) (**f**) in not infected (NI) fish exposed to 0 % of GLs (NI-C) and an extreme

871 dose of GLs (NI-13) and infected fish (I) fed feed with 0 % of GLs-containing raw ingredient (I-

872 C), 3.6 % (I-3.6) and 13 % (I-13). Blood plasma profiling was performed on 15 individuals from

873 NI-C and 16 individuals from NI-13 in Trial 1, and 9 fish from each of the groups in Trial 2.
874 Asterisks shown between NI and I groups refer to statistical differences of NI-C and NI-13 as
875 one group *vs* I-C, I-3.6 and I-13 as the other group. Asterisks shown above bars denote significant
876 differences between two groups. $*P < 0.05$, $**P < 0.01$.
877
878 **Fig. 2**
879



880
881
882 **Fig. 2 a** Scoring of liver steatosis by light microscopy in Trial 1, 2 and 3. Liver sections from
883 Trial 1 (NI-C and NI-13), Trial 2 (I-C, I-3.6 and I-13) and Trial 3 (I-C and I-2) were scored from
884 0 to 5 [88], based on the degree of vacuolization in the cytoplasm and the degree of distribution
885 of the vacuolated hepatocytes (Additional file 1: Table S2). 4–6 fish in each group were analyzed.
886 Solid black line shows the mean score in each group, and black dots show the individual fish
887 scores. **b** Exemplary images of livers showing different level of steatosis. **c** Micrograph of a fish

from group I-3.6 fish (Trial 2) with a score of 0. **d** Micrograph of a fish from group NI-C fish from Trial 3 with a score of 1. **e** Micrograph of a fish from group I-C fish from Trial 2 with a score of 3. *Scale-bars*: 100 µm

Fig. 3

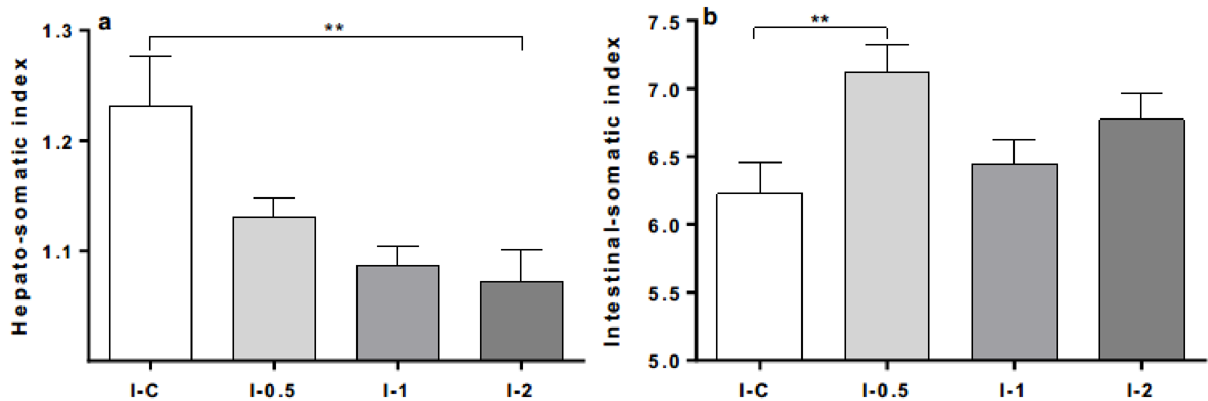
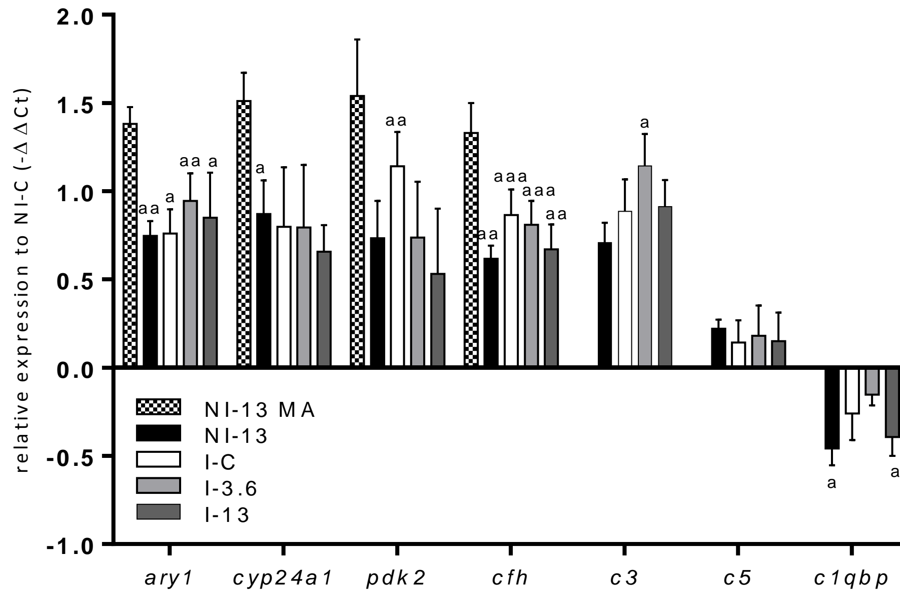


Fig. 3 Organ indices in lice infected fish (I) fed inclusion levels of 0 (I-C), 0.5 (I-0.5), 1 (I-1) and 2 % (I-2) of GLs. **a** Hepato-somatic indices (HSI). **b** Intestinal-somatic indices (ISI). Number of fish in each group is 10. Asterisks denote level of significance between groups: $**P < 0.01$. ISI data was analyzed by One-way ANOVA followed by Tukey's multiple comparisons test, while HSI data was analyzed by the Kruskal-Wallis test followed by the *post-hoc* Dunn's test

912 **Fig. 4**

913



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915

916 **Fig. 4** Hepatic gene expression of *ary1*, *cyp24a1* and *pdk2* with roles in metabolic adaptation to
917 nutrient availability, complement regulator *cfh*, complement components *c3* and *c5*, and comple-
918 ment regulator *c1qbp* measured by qPCR and shown as mean -ΔΔCt. The first bar for each gene
919 shows the logER value measured by microarray in not infected (NI) fish ($n = 5$) fed 13 % of the
920 GLs-containing raw ingredient (NI-13). Gene expression in infected (I) fish fed increasing levels
921 of GLs-containing raw ingredient, 0 (I-C), 3.6 (I-3.6) and 13 % (I-13), were measured by qPCR.
922 The zero is set to NI fish fed 0 % dietary GLs (NI-C). Number of fish in each group is 9. The
923 letter "a" denotes significant expression difference to NI-C, "aaa" when $P < 0.001$, "aa" when P
924 < 0.01 and "a" when $P < 0.05$

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932 **Fig. 5**

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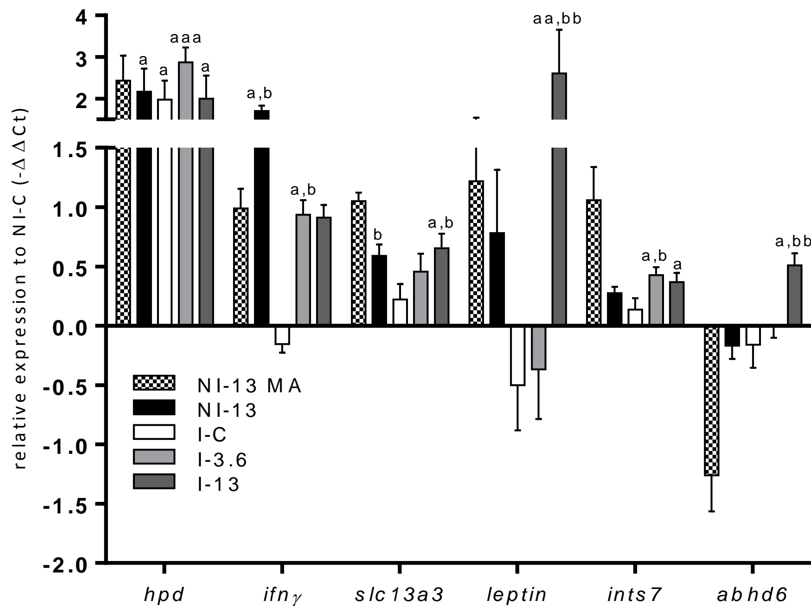

934

935 **Fig. 5** Renal gene expression of *hpd* from the tyrosine breakdown pathway, anti-fibrotic *ifnγ*.
936 *slc13a3* involved in the maintenance of citrate levels, pro-fibrotic *leptin*, the DNA damage re-
937 sponse gene *ints7*, and *abhd6* with functions in the regulation of lipogenesis in kidney, measured
938 by qPCR and shown as mean -ΔΔCt. The first bar for each gene shows the logER value measured
939 by microarray in not infected (NI) fish (n = 5) fed feed with 13 % inclusion level of GLs-con-
940 taining raw ingredient (NI-13). Gene expression in infected (I) fish fed increasing levels of GLs-
941 containing raw ingredient, 0 (I-C), 3.6 (I-3.6) and 13 % (I-13), were measured by qPCR. The zero
942 is set to NI fish fed 0 % dietary GLs (NI-C). Number of fish samples in each group is 9. The letter
943 "a" denotes significant expression difference to NI-C, and "b" denotes significant expression
944 difference to I-C, "aaa" when $P < 0.001$, "aa/bb" when $P < 0.01$ and "a/b" when $P < 0.05$

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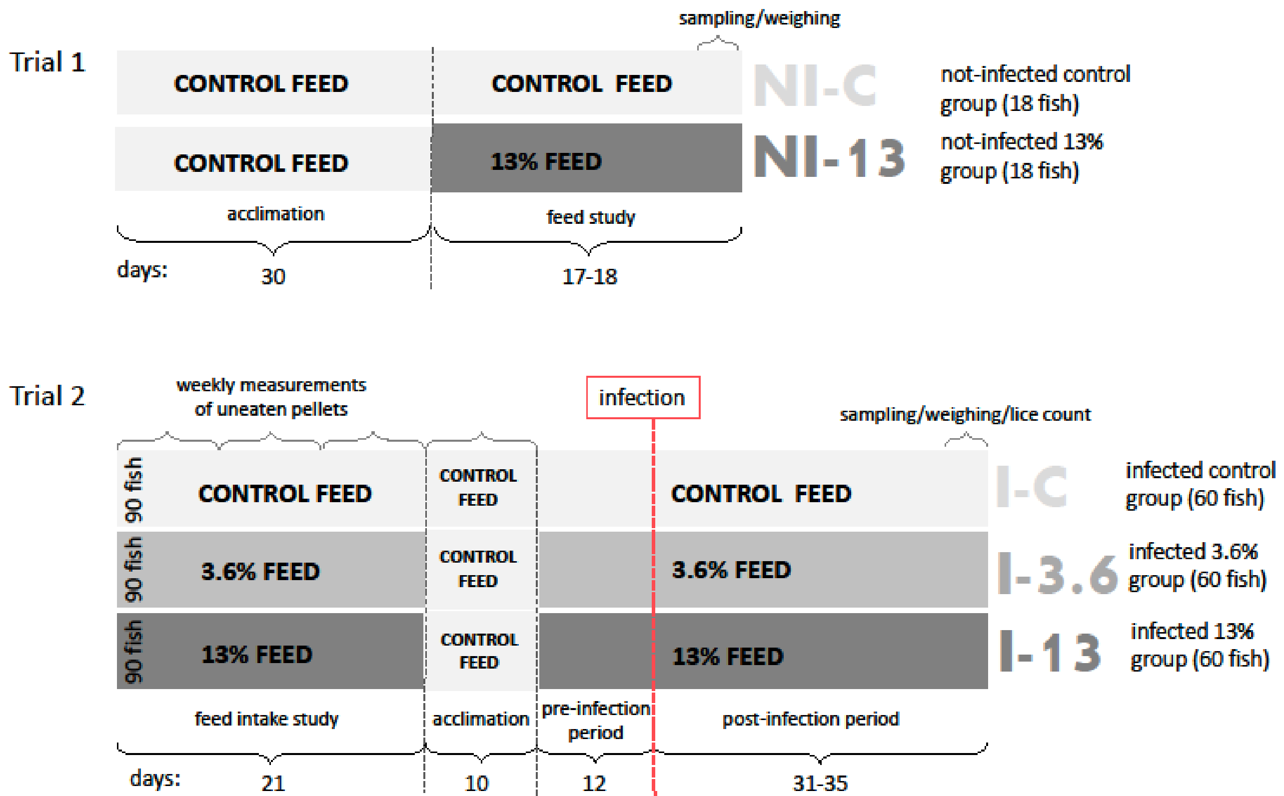
952 **Fig. 6**



953

954 **Fig. 6** Experimental setup of Trial 1 and Trial 2 (modified from [17]). **a** Trial 1. Feed study. To
955 study responses of the feed (without infection), Atlantic salmon were fed control feed and high
956 level (13 %) of GLs-containing raw ingredient. All fish received control feed for 30 days during
957 the acclimation period. Sampling of tissues and weighing of the fish from two groups [not-in-
958 fected control group (NI-C) and not-infected 13 % group (NI-13)] were performed 17–18 days
959 after feeding experimental feeds. **b** Trial 2. Feed intake and lice challenge study. Atlantic salmon
960 were exposed to feeds containing 0, 3.6 and 13 % of GLs for 21 days. Control feed was then fed
961 for 10 days during the acclimation period. The trial continued with fish exposed to control, and
962 3.6 and 13 % GLs feeds for 12 days (pre-infection period). The tissue sampling, weighing and
963 lice counting from the three dietary groups [infected fish fed control feed (I-C), infected fish fed
964 3.6 % GLs feed (I-3.6) and infected fish fed 13 % GLs feed (I-13)] challenged with *L. salmonis*
965 (50 copepodids per fish) were accomplished after 31–35 days of infection (post-infection period)

966

967 **Table 1** Mean weight ± standard deviation (SD) and mean condition factor ± SD in not infected fish
968 exposed to 0 % of the GLs-containing raw ingredient (NI-C) and 13 % (NI-13) in Trial 1. Data was
969 analyzed by *t*-test.

970

Trial 1	NI-C (*n* = 18)	NI-13 (*n* = 18)
Weight[a] (g)	825.6 ± 117.5	805 ± 139
CF[b]	1.52 ± 0.08	1.56 ± 0.08

971 [a]t-test: $t_{(34)} = 0.49$, $P = 0.62$.
972 [b]Condition factor was calculated by the formula (weight*100/length^3) for each individual fish.
973 [b]t-test: $t_{(34)} = 1.37$, $P = 0.18$.

974

975 **Table 2** Mean weight ± standard deviation (SD) and condition factor ± SD in *L. salmonis*-infected
976 fish exposed to 0 % of the GLs-containing raw ingredient (I-C), 3.6 % (I-3.6) and 13 % (I-13) in Trial
977 2. Data was analyzed by One-way ANOVA.

978

Trial 2	I-C (*n* = 60)	I-3.6 (*n* = 60)	I-13 (*n* = 60)
Weight[a] (g)	871 ± 127	751 ± 121****	726 ± 113****
CF[b]	1.43 ± 0.13	1.54 ± 0.16****	1.52 ± 0.13***

979 [a]ANOVA: $F_{(2,177)} = 24.86$, I-C *vs* I-3.6 $P < 0.0001$, I-C *vs* I-13 $P < 0.0001$.
980 [b]Condition factor was calculated by the formula (weight*100/length^3) for each individual fish.
981 [b]ANOVA: $F_{(2,177)} = 11.37$, I-C *vs* I-3.6 $P < 0.0001$, I-C *vs* I-13 $P < 0.001$.
982 ***$P < 0.001$, **** $P < 0.0001$: significant differences in comparisons with control.

983

984 **Table 3** Mean weight ± SD and condition factor ± SD in *L. salmonis* infected fish exposed to 0 % of
985 the GLs-containing raw ingredient (I-C), 0.5 % (I-0.5), 1 % (I-1) and 2 % (I-2) in Trial 3.

986

Trial 3	I-C (*n* = 78)	I-0.5 (*n* = 76)	I-1 (*n* = 72)	I-2 (*n* = 73)
Weight[a]	540 ± 110	554 ± 103	540 ± 119	553 ± 103

CF[b]	1.2 ± 0.08	1.2 ± 0.08	1.21 ± 0.07	1.2 ± 0.05

987 [a]ANOVA: $F_{(3,292)} = 0.4$, $P = 0.75$

988 [b]Condition factor was calculated by the formula (weight*100/length^3) for each individual fish.

989 [b]ANOVA: $F_{(3,291)} = 1.85$, $P = 0.14$.

990

991 **Table 4** Near infrared spectroscopy (NIR) of Norwegian quality cut (NQC) samples from Trial 3.
992 Lice infected (I) fish were fed inclusion levels of 0 % GLs-containing raw ingredient (I-0), 0.5 % (I-
993 0.5), 1 % (I-1) and 2 % (I-2). Levels of 16:0, 18:1, 22:6n-3 are shown, as they were the only parameters
994 that were significantly different in the One-way ANOVA or Kruskal-Wallis test.

995

Trial 3	14:0[a]	16:0[b]	18:1[c]	22:6n-3[d]
	(n = 10)	(n = 10)	(n = 10)	(n = 10)
I-0.5	3.23 ± 0.046**	15.85 ± 1.16**	29.18 ± 1.19	7.52 ± 0.63**
I-1	3.311 ± 0.067	14.94 ± 1.5	29.49 ± 2.4	7.80 ± 0.61
I-2	3.373 ± 0.059	15.82 ± 1**	28.01 ± 2*	7.78 ± 0.66*
I-C	5.777 ± 1.547	12.67 ± 4.23	23.47 ± 8.4	8.64 ± 0.81

996 [a]14:0 Kruskal-Wallis H-test: $\chi^2 = 7.8$, $df = 3$, $P = 0.01$.

997 [b]16:0 Kruskal-Wallis H-test: $\chi^2 = 7.8$, $df = 3$, $P = 0.027$; I-0.5 *vs* I-C $P = 0.0096$; I-2 *vs* I-C $P =$
998 0.009.

999 [c]18:1 Kruskal-Wallis H-test: $\chi^2 = 7.8$, $df = 3$, $P = 0.03$.

1000 [d]22:6n-3 ANOVA: $F_{(3,34)} = 4.5$; I-0.5 *vs* I-C $P = 0.0064$; I-2 *vs* I-C $P = 0.05$).

1001 *$P < 0.05$, ** $P < 0.01$: significant differences in comparison with control.

1002

1003 **Table 5** Differentially expressed genes in the liver of not infected (NI) salmon fed an extreme dose of
1004 GLs-containing raw ingredient (NI-13) in comparison to NI salmon fed 0 % dietary GLs (NI-C). Data
1005 are log2-ER.

1006

Negative regulation of proliferation	log2-ER
Cullin 1b	1.82
Btg1	1.66
Costars family protein abracl	1.61
NOD-like receptor C	1.42
HIV-1 Tat interactive protein 2	1.01
Nitrilase homolog 2	0.90

Positive regulation of proliferation	
Transmembrane protein 53	-1.17
Cell division cycle protein 23 homolog	-1.20
Cyclin-A2	-1.21
Arntl2 protein	-1.23
Ornithine decarboxylase 1	-1.29
G2/mitotic-specific cyclin-B1	-1.47
Placenta-specific gene 8 protein	-1.62
Chromatide segregation and chromosome organization	
Haspin, hasp	-1.03
N-acetyltransferase esco1	-1.20
Securin	-1.65
DNA replication	
Ribonucleoside-diphosphate reductase large subunit	-1.03
Chromatin regulation	
Histone deacetylase 2	-1.00
DnaJ homolog subfamily C member 2	-1.06
Lamin B receptor	-1.21
Condensin complex subunit 3	-1.56
DNA damage and repair	
Uracil-DNA Glycosylase	-1.06
Ubiquitin carboxyl-terminal hydrolase isozyme L5	-1.18
Biotransformation/detoxification	
Cytochrome P450 24A1, mitochondrial precursor	1.51
Glucocorticoid receptor	1.51
Arylamine N-acetyltransferase, pineal Gland isozyme NAT-10	1.38
Transcription factor jun-B	1.25
Glutathione S-transferase 3	1.00
Glutathione S-transferase theta-1	0.93
Epoxide hydrolase 1	0.85
Epoxide hydrolase 2 cytoplasmic	0.92
UDP Glucuronosyltransferase 1 family polypeptide b7 short isoform	0.81
Liver function	
CMP-sialic acid transporter	2.00
Pyruvate dehydrogenase kinase isozyme 2, mitochondrial precursor	1.54
Bile salt export pump	1.34
Hydroxyacid-oxoacid transhydrogenase, mitochondrial precursor	-1.08
Novel protein similar to vertebrate scavenger receptor protein	-1.60
Iron metabolism	

Hepcidin-1	1.60
Cytochrome b reductase 1	1.29
Porphobilinogen deaminase	-0.98
Probable cytosolic iron-sulfur protein assembly protein ciao 1	-0.92
Complement immune response	
Complement factor H precursor	1.33
Properdin P factor 2	1.04
Properdin P factor 3	0.94
Complement C1q-like protein 4	0.88

1007

1008 **Table 6** Differentially expressed genes in distal kidney of not infected (NI) salmon fed an extreme
1009 dose of GLs-containing raw ingredient (NI-13) in comparison to NI salmon fed 0 % dietary GLs (NI-
1010 C). Data are log2-ER

1011

Biotransformation, detoxification	**log2-ER**
Solute carrier family 22 member 2	1.41
NAD(P)H dehydrogenase quinone 1	1.26
Serine--pyruvate aminotransferase, mitochondrial precursor	1.24
Glutamate-cysteine ligase catalytic subunit	0.95
Epoxide hydrolase 2	-0.90
Oxidation-reduction processes	
Sarcosine dehydrogenase	1.11
Ubiquinol-cytochrome c reductase core I protein	1.02
Cytochrome B	-0.94
Regulation of fibrosis and kidney stone formation, protection from injury	
4-hydroxyphenylpyruvate dioxygenase	2.43
Deltex-3-like	1.32
Leptin	1.22
Solute carrier family 13 member 3	1.05
Fetuin-A	1.03
Interferon γ	0.99
Ski-interacting protein	0.97
Sulfide quinone reductase-like (Yeast)	-1.08
Serine/threonine/tyrosine-interacting protein	-1.15
Sparc precursor	-1.17
Relaxin-3	-2.01
Extracellular matrix components and regulation	

Fibrinogen alpha chain	1.07
Wnt-5b	1.06
Collagen a3(I)	0.98
Microfibrillar-associated protein 1	0.86
Transforming growth factor, beta (TGFβ)- receptor III, TGBR3	-1.02
Transforming growth factor beta (TGFβ)-1-induced transcript 1 protein	-1.14
Proteolysis	
Matrix metalloproteinase 9	1.15
OTU domain-containing protein 6B	0.90
Prepro-cathepsin C	0.86
Proteasome subunit alpha type-1	0.83
Procollagen C-endopeptidase enhancer 1	-0.92
N-acetylated alpha-linked acidic dipeptidase-like 1	-1.01
ADAMTS15	-0.88
Iron homeostasis	
Sideroflexin-2	0.89
Sideroflexin-4	0.84
Ferritin, middle subunit	0.83

1012

1013 **Table 7** Differentially expressed genes in the muscle of not infected (NI) salmon fed an extreme dose
1014 of GLs-containing raw ingredient (NI-13) in comparison to NI salmon fed 0 % dietary GLs (NI-C).
1015 Data are log2-ER

1016

Positive regulation of proliferation	**log2-ER**
Ccr4-not transcription complex subunit 6	1.13
Placenta-specific gene 8 protein	0.98
Haspin	-1.08
Negative regulation of proliferation, apoptosis	
Actin-related protein 2/3 complex subunit 1B	1.35
Kruppel-like factor 11	1.26
Androgen-induced proliferation inhibitor	1.21
Tumor necrosis factor receptor superfamily member 1A	0.88
Bax	0.86
Caspase-activated DNase	0.82
Cyclin-D-binding Myb-like transcription factor 1	-0.84
DNA replication	
Nuclear factor 1	1.31

DNA replication licensing factor mcm3	-0.83
DNA replication licensing factor mcm5	-1.11
DNA damage and repair	
E3 sumo-protein ligase nse2	-0.88
TFIIH basal transcription factor complex helicase XPB subunit	-0.89
FACT complex large subunit	-1.00
Ubiquitin-conjugating enzyme E2 T	-1.43
Nucleotide metabolism	
Adenylosuccinate synthetase isozyme 2	-0.98
Deoxycytidylate deaminase	-1.22
Muscle metabolism, myogenesis	
Acta1 protein	1.26
Microtubule-associated protein 1 light chain 3 alpha	1.17
Tetranectin	1.07
Protein arginine methyltransferase 5	1.03
Myosin 1	1.00
Tripartite motif-containing 55b	0.84
Four and a half LIM domains protein 1	-0.81
Heparin-binding growth factor 1	-0.85
Myosin Va	-1.03
Sodium/hydrogen exchanger	-1.45
Myotubularin	-1.93
Negative regulation of myogenesis	
Cardiomyopathy associated 5 like	1.29
Stathmin	1.03
Histone deacetylase 4	-1.66
Biotranformation, detoxification	
6-phosphogluconolactonase	1.21
Glutathione transferase omega-1	1.10
Cytochrome b-c1 complex subunit 6, mitochondrial	1.01
Alcohol dehydrogenase class-3	0.84
Aryl hydrocarbon receptor 2 beta	-0.86
Cytochrome P450 1A1	-0.86
Iron metabolism	
Heme oxygenase 1	1.69
Proton-coupled folate transporter	1.29
NADH-cytochrome b5 reductase 1	0.94
Uroporphyrinogen decarboxylase	0.84
NADPH-dependent diflavin oxidoreductase 1	-0.96

Sideroflexin-2	-1.17

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1018 **Additional files**

1019 **Table S1.** An overview over the various samples and analysis methods applied in Trial 1, 2 and 3.

1020 Blank spaces indicates that the analysis were not included in the trial.

Trial/analysis method	Trial 1	Trial 2	Trial 3
Groups	Not infected control (NI-C) Not infected 13 % (NI-13)	Infected control (I-C) Infected 3.6 % (I-3.6) Infected 13 % (I-13)	Infected control (I-C) Infected 0.5 % (I-0.5) Infected 1 % (I-1) Infected 2 % (I-2)
Microarray	Liver, distal kidney, muscle		
qPCR	Liver, distal kidney	Liver, distal kidney	
Weights/condition factor	Yes	Yes	Yes
Organo-somatic indices			Hepato-somatic index (HIS) Intestinal-somatic index (ISI)
Liver steatosis scoring	Yes	Yes	Yes
Near infrared spectroscopy (NIR)			Norwegian quality cut (NQC)
Biochemical and enzyme plasma profiling	Yes	Yes	

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1022 **Table S2.** Scoring of liver steatosis in individual sections based on the following system described in
1023 L. Martinez-Rubio *et al*. 2013 (88)

0 Formation of vacuoles in the cytoplasm, involving less than 10% of the hepatocytes and including less than 25% of the area of the individual hepatocytes
1 Formation of vacuoles in the cytoplasm, involving less than 25% of the hepatocytes and including less than 25% of the area of the individual hepatocytes
2 Formation of vacuoles in the cytoplasm, involving less than 50% of the hepatocytes and including less than 50% of the area of the individual hepatocytes
3 Formation of vacuoles in the cytoplasm, involving less than 75% of the hepatocytes and including less than 75% of the area of the individual hepatocytes
4 Formation of vacuoles in the cytoplasm, involving less than 90% of the hepatocytes and including less than 80% of the area of the individual hepatocytes
5 Formation of vacuoles in the cytoplasm, involving more than 90% of the hepatocytes and including more than 80% of the area of the individual hepatocytes

1031 **Table S3.** Primers used for qPCR analyses

Gene name and symbol	**Accession**	**Size**	**Primers**
Arylamine N-acetyltransferase (*ary1*)	BT046633.1	75	F:GCTTGGGTGCTAAAA-GAGA
			R:CTGGTTGATGGTGTT-GTTGT
Abhydrolase-domain containing protein 6 (*abhd6*)	NM001140355.1	112	F:ATCCCTCTGATCCCCTC-TAC
			R:CTCGAACATCCAC-CAATCCC
Complement factor H-like (*cfh*)	XM014123545.1	119	F:TGCCGAACATAAGGATC ACA
			R:ATTGG-CAATGAGGCAAGTTC

Complement component 1Q binding (*c1qbp*)	CA387557	217	F:CGGTCTCTCTG-GATGATGAGCCATA
			R:CCACATCCACAC-GACACAGGAGTA
Cytochrome P450 24A1 (*cyp24a1*)	BT059557.1	78	F:ACATCTACCGCCACAG-TCA
			R:TCTCCACTCCTCCGATCT
Complement C3 (*c3*)	L24433.1	106	F:GAG-GAAAGGTGAGCCAGATG
			R:TGTGTGTGTCGTCAGCTT CG
Complement component 5 (*c5*)	XM014174798.1	121	F:AAGGCCAGTTGCAG-TTCTGT
			R:CCTGGGAATCCAAAGGG-TAT
4-hydroxyphenylpyruvate dioxygenase (*hpd*)	NM001140426.1	184	F:TTGATGAAGCATGGG-GATGGG
			R:TGAGAGTGTGTGTT-GTATCGCC
Elongation factor 1 alpha (*ef1α*)	BT072490.1	88	F:GCTGTGCGTGACATGAG G
			R:ACTTTGTGACCTTGCCGC
Interferon γ (*ifnγ*)	AY795563	159	F:CTAAAGAAGGACAACCG-CAG
			R:CACCGTTAGAGGGA-GAAATG
Integrator complex subunit 7 (*ints7*)	XM014204660.1	175	F:ACAACCAGCAGCAG-CAACA

			R:GCTCCAGTCCAG-TCTTTTCAAA
Leptin	FJ830677.1	101	F:CTCCTGTT-GTCCTCTCTGT
			R:ATGGTTTGAG-CAAGGTCTTT
Pyruvate dehydrogenase kinase isozyme 2 (*pdk2*)	BT059601.1	151	F:AAGGTGATGGA-TAGGGGTG
			R:AGAGGCGTGAAATGGGA
Solute carrier family 13 member 3-like (*slc13a3*)	BT058859.1	130	F:ACAGGACGAAAGAG-CACAA
			R:CAGGAGAACGG-CAAACAAAA

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